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Intercellular Adhesion in Resin Canal Tissue Isolated from Slash Pine Chlorite Holocellulose

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INTERCELLULAR ADHESION IN RESIN CANAL TISSUE ISOLATED FROM SLASH PINE CHLORITE HOLOCELLULOSE

A thesis submitted by

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SUMMARY

Resin canal tissue was isolated from a slash pine (Pinus elliottii Engelm.) chlorite holocellulose in sufficient quantity to allow both chemical analyses and structural examination to be made. The effects of selected chemical treatments on cell separation, and the chemical composition and structure of the cell walls and the middle lamella of the isolated canal tissue (canal complex) were studied with respect to intercellular adhesion. The problem was approached with emphasis on characterization of the middle lamella with respect to boundaries, structure, and chemical composition. Chemical and physical bonding phenomena were investigated indirectly. The major portion of the experimental work involved structural studies and chemical analyses of the canal complex before and after soaking in solutions of ferric chloride, hydrochloric acid, or potassium ferrocyanide. Structural aspects of the treated tissue were evaluated with the electron microscope using surface replicas and stained and shadowed, ultrathin sections.

The slash pine canal complex is a thin-walled, unlignified tissue which appears to be without secondary thickening. The mature structure consists of a central canal surrounded by three cellular layers. Epithelium forms the inner layer while the middle and outer layers consist of short (intermediate) and elongated (outer) parenchyma cells, respectively. The epithelia are the largest cells with a tightly compacted wall adjacent to the canal and a laminated and thick wall next to the intermediate cells. Intermediate cells are cubelike and are smaller than the epithelial cells. Outer cells are three times as long and slightly wider than the intermediate cells. The intermediate and outer cell walls, as well as epithelial cell walls adjacent to intermediate cells, have a similar structure. The three cell types vary with respect to their formation from fusiform cambial initials and the number of layers in the mature wall. Pit fields are found in the walls of all cell types while end wall perforations are observed only in intermediate and outer cells.

The middle lamella is defined as the region between two adjacent primary cell walls, while the primary wall is that structure which encloses a protoplast in the mature canal complex. In contrast to previous concepts, the middle lamella is a complex structure which can contain intercellular membranes and cellulosic and non-cellulosic (probably polygalacturonan) intercell-wall fibrils. Intercellular membranes encapsulate two or more cells and consist of one or more intact ancestral walls. The membranes are definitely not confined to crossing intercellular interfaces. The chemical composition of the ancestral walls is changed when they are penetrated and surrounded by substances characteristic of the middle lamella. This must occur as the ancestral walls are expanded during cell division and cell elongation. The number and total thickness of ancestral walls in the mature middle lamella varies with the number of cell divisions and the location of the cell plate(s) within a fusiform cambium daughter cell. Cellulosic fibrils connect adjacent ancestral and primary walls, adjacent ancestral walls, and adjacent cell walls. Noncellulosic fibrils have a distinct structure, are found throughout the middle lamella, and probably penetrate and connect ancestral walls. They may also connect adjacent ancestral and primary walls. In contrast to the slightly directed cellulosic fibrils, the noncellulosic structures appear to be randomly oriented. Development of the canal, and of the ray tissue associated with the canal complex, can be traced by studying cell wall ultrastructure.

The middle lamella is easily distinguished from the primary cell wall by its high calcium content and texture. Chemical analyses show that canal complexes treated with hydrochloric acid or potassium ferrocyanide have similar compositions. The treatments remove most of the calcium and pectic substances (including noncellulosic fibrils) from the middle lamella and leave networks of cellulose fibrils contaminated with hemicelluloses. Because only a very small quantity of the hemicelluloses are extracted by the treatments, they are probably closely associated with the cellulose

fibrils of the middle lamella and the primary wall. The middle lamella contains ancestral walls and cellulosic and noncellulosic fibrils embedded in an amorphous calcium pectate matrix, while the primary wall is a more compact structure containing fewer pectic substances. Adjacent cells separate from one another along cleavage planes within the middle lamella or along the middle lamella — primary wall interface. No single location appears to be preferred.

Although chemical and physical bonding forces were only investigated indirectly, new conclusions were reached regarding the role of multivalent cations in intercellular adhesion. The major function of multivalent cations, such as calcium, in the isolated canal tissue is to stabilize and prevent solution of the acidic components of the middle lamella and primary cell wall. Removal of the calcium and most of the pectic substances does not produce substantial cell separation unless the treated tissue is soaked in an aqueous medium of approximately zero ionic strength. When canal complexes are treated with potassium ferrocyanide and then soaked in water, adjacent cell walls are separated. This phenomenon is attributed to the development and expansion of electrical double layers on the surfaces of wall elements in the canal tissue. The presence of intercellular membranes and inter-cell-wall fibrils (cellulosic) prevents complete cell separation. Residual hemicelluloses and acidic substances in treated canal complexes may be organized into polymeric networks which surround and connect adjacent fibrils. However, substances which contain organic nitrogen apparently have an insignificant role in intercellular adhesion.

Bonding sites are continuous and nonspecific throughout the middle lamella and the primary cell wall. Separated cells of canal tissue, untreated or soaked in acid or potassium ferrocyanide, are readhered by addition of ferric, calcium or uranyl ions provided adjacent cell walls are in close proximity. This effect is

primarily attributed to chemical rather than physicochemical phenomena. In addition, both separated tracheids and intact canal complexes can be bonded together provided they are in close proximity. Preliminary studies indicate that the ability to bond wood fibers with multivalent cations may be particularly significant with respect to increasing the wet strength of paper.

INTRODUCTION

Although the problem has been studied extensively, the substances involved and the location and mechanism(s) of intercellular adhesion in thin-walled, unlignified plant tissue have not been determined. This deficiency is largely due to an inability to define the boundaries, structure, and chemical composition of the middle lamella. For this reason, current concepts of intercellular adhesion in plant tissues are based essentially on intelligent speculation. Reviews by Roelofsen (1, 2), Esau (3), Wardrop (4), and Albersheim (5) adequately describe current knowledge of the middle lamella and the cell wall. The middle lamella is described as an amorphous, isotropic substance with calcium pectate being the major constituent. Pectic substances have plastic, highly hydrophilic, and colloidal properties. The quantities of nonpectic substances in the middle lamella (carbohydrate and nitrogen-containing materials, etc.) are unknown. Albersheim (5), in contrast to the other reviewers (1-4), does not consider that the middle lamella is necessarily isotropic.

Before the mechanism(s) of intercellular adhesion is investigated, the cell wall and middle lamella should be characterized with respect to boundaries, structure, and chemical composition. As this has not been achieved in previous studies, acceptable conclusions have not been reached. Ginzberg (6) presented one of the more complete studies on intercellular adhesion. The effects of sequestering and protein denaturing reagents on an immature plant tissue were evaluated with respect to the degree of cell separation. Data from this study supported the conclusion that, in addition to pectic substances, a protein gel structure cross-linked by metal cations was involved in intercellular adhesion. This conclusion would be more acceptable if the effect of the reagents had also been related to tissue structure and chemical composition. Letham (7) also considered the problem with respect to sequestering reagents, and his data both support and contradict Ginzberg's conclusions (2). Wyn

Jones and Lunt (8) and Taylor and Wain (9) discussed the effect of sequestering reagents with reference to calcium pectate and cell elongation. Taylor and Wain (9) found that complexing reagents extract considerable quantities of pectic substances as well as multivalent cations from immature plant tissues. The fact that sequestering reagents extract pectic and possibly other substances sheds further doubt on Ginzberg's conclusions. Although Ginzberg must have been aware that treatment changed the tissue, no attempt was made to measure the extent of the change.

The role of physicochemical forces in intercellular adhesion has been practically ignored in previous studies. Pethica (10), in a theoretical account of the physicochemical aspects of intercellular adhesion, describes nine possible forces of adhesion and three of repulsion. Armstrong (11) considered the role of metal cations with respect to their effect on surface charge. Although these articles were written with reference to animal cells, the principles discussed are applicable to plant material. Any study of intercellular adhesion, to be complete, must consider physical and chemical bonding forces simultaneously with tissue structure and chemical composition.

APPROACH TO THE PROBLEM

It is impossible in any one study to determine the structural, chemical, and physical phenomena involved in intercellular adhesion. In any experimental program, only one of these three can be emphasized. Hence, a program was designed to characterize the middle lamella with particular reference to boundaries, structure, and chemical composition. Chemical and physical bonding forces were investigated indirectly. The investigation was oriented toward structure, as definition of the middle lamella was considered essential before an understanding of intercellular adhesion could be reached. The approach is outlined in Fig. 1.

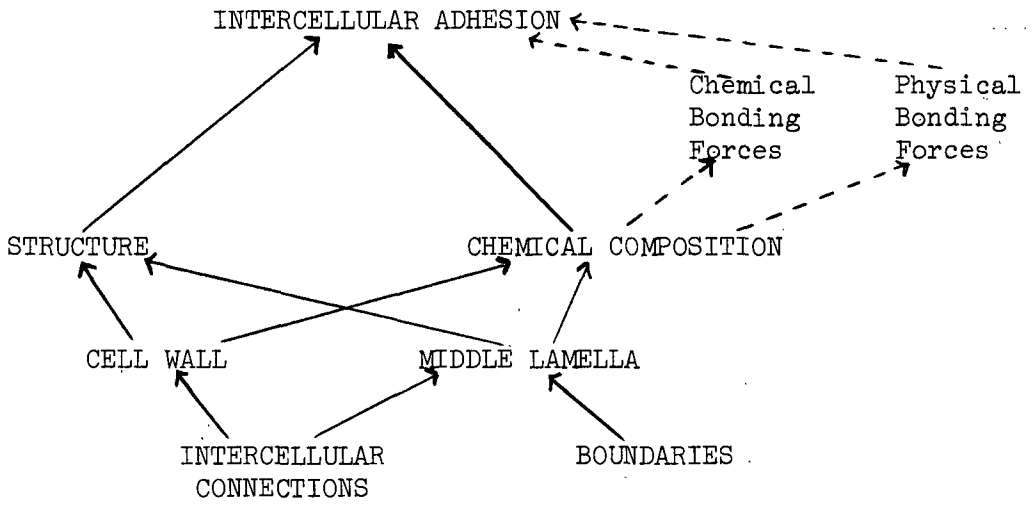


Figure 1. Schematic Outline of the Areas Studied

Plant tissue was subjected to a variety of selected chemical treatments, each producing a different degree of cell separation. Hence, structures masked in untreated, and absent in separated tissue, were observed in partially separated material. The effects of each treatment on the plant tissue were evaluated with respect to the following:

1. The chemical and physical properties of the reagent.
2. The degree of cell separation.
3. The extent to which partially and completely separated cells could be readhered.
4. The chemical composition of the tissue residue and the residual treating liquor.
5. The middle lamella and cell wall ultrastructure.
6. The physical and/or chemical phenomena introduced into or removed from the tissue as a result of chemical treatment.

SELECTION OF PLANT MATERIAL

As the investigation was primarily designed to characterize the middle lamella, the plant material studied had to be suitable for both structural and chemical

analyses. The following properties were considered important in the selection of the plant tissue:

1. Isolation in sufficient quantity to allow chemical composition and structural analyses to be made with respect to each chemical treatment.

2. Suitability for both light and electron microscopic evaluation. A thin-walled tissue was required to ensure that ultrathin sections were readily obtainable.

3. Identifiable tissue axis, to ensure that structural data could be related to the intact plant.

4. Maturity, to ensure that variation due to cell elongation and/or cell expansion did not affect the chemical and structural data.

Thompson, et al. (14-16) isolated, intact from a number of pine holocelluloses, the thin-walled tissue which surrounds the resin canals. Cells of the isolated tissue were partially dissociated when subjected to treatment by complexing reagents, and preferential cell separation occurred in the direction of the canal axis (16). When certain properties of this resin canal tissue were compared with those of immature and mature holocellulose, little similarity was noted (17). However, when the properties described by Thompson, et al. (15-17) were compared with the data of Ginzberg (6) and Letham (7), the similarities were remarkable.

Resin canal tissue was selected as a suitable material for study with respect to the previously listed requirements. However, many of the chemical and physical phenomena in the untreated and unisolated canal tissue were irreversibly changed by the delignification process. These unidentifiable changes were insignificant when the approach to the study was considered. Any conclusions reached regarding the chemical and physical bonding forces in the isolated canal tissue must be carefully examined before they are applied to living plant material. The fact that the canal tissue was isolated from pulped wood is significant, as intercellular adhesion and cell separation are of considerable importance to the paper and allied industries.

SPECIFIC AIMS OF THE STUDY

Intercellular adhesion was investigated by characterizing the middle lamella with respect to boundaries, structure, and chemical composition. The following questions and alternative answers were used in evaluation of the middle lamella:

1. What are the boundaries of the middle lamella?

   a. The middle lamella is an adhesive layer between adjacent cell walls and, hence, has distinct boundaries.

   b. The surfaces of adjacent cell walls are in contact, and the middle lamella, as such, does not exist. The observed concentration of pectic substances (1-5) is due to their presence at the interface and in the outer layer(s) of the cell walls.

   c. No distinct boundaries exist between the cell wall and the middle lamella. The cell wall surface is not smooth or definable but is continuous with the middle lamella.

2. Does the middle lamella contain distinct structures embedded in an amorphous matrix, or is it an amorphous isotropic layer?

3. What is the chemical composition of the middle lamella?

   a. The middle lamella consists almost entirely of pectic and inorganic materials.

   b. In addition to pectic and inorganic materials, the middle lamella contains other substances in significant quantities (carbohydrates and organic nitrogen).

The above questions were analyzed further with reference to intercellular adhesion. The following questions and alternative answers were considered relevant:

1. What structures connect adjacent cell walls, and what is their role in intercellular adhesion?

   a. Plasmodesmata (3) and/or the associated primary cell wall and middle lamella structure (pit-field complex) may have a significant role.

   b. Intercellular membranes (13) may have a significant role.

   c. Intercell-wall fibrils may exist and may have a significant role.

2. What are the physical and/or chemical bonding forces involved in intercellular adhesion? Due to the approach to the study, this question can only be answered generally.

   a. Calcium pectate is the only identifiable source of bonding.

   b. In addition to calcium pectate, other forms of physical and/or chemical bonding are identifiable.

3. What are the sites of physical and/or chemical bonding within the middle lamella, the cell wall, and along the cell wall-middle lamella interface?

   a. Bonding sites are restricted to specific nonrandom locations.

   b. Bonding sites are randomly scattered.

4. Why does preferential cell separation occur in the direction of the canal complex axis?

   a. Transverse and longitudinal intercellular regions have different chemical compositions.

   b. Transverse and longitudinal intercellular regions have different structures connecting adjacent cells.

EXPERIMENTAL

ISOLATION OF THE RESIN CANAL TISSUE

A slash pinewood bolt, taken from the stem of a 26-year-old tree, was the source of canal tissue. A chlorite holocellulose was prepared, with slight modification, by the method of Thompson, et al. (15, 16) (Appendix I). New isolation and purification procedures were developed to ensure sufficient tissue for both chemical and microscopic evaluation (Appendix II). No attempt was made to separate earlywood and latewood or heart- and sapwood canal tissue. Although an independent study was not made, little variation between longitudinal canal complexes was observed. Isolated longitudinal tissue was distinguished from the radial structures by its greater diameter and the location and orientation of associated ray and fiber debris. Only longitudinal tissue was used in the microscopic and cell separation studies, but both longitudinal and radial structures were included in the material subjected to chemical analyses. The radial structures were much smaller than the longitudinal and made up less than 2% of the total canal tissue.

Three batches of chlorite holocellulose were prepared at room temperature. Canal tissue isolated from two of the batches was used to determine chemical composition with respect to a variety of chemical treatments, and material from the other was used in the microscopic and cell separation studies. A sample of canal tissue (untreated) from the third batch was also subjected to chemical analyses.

CHEMICAL TREATMENTS AND ANALYSES

Three chemical reagents were used throughout the investigation because they formed a series with respect to their ability to separate canal tissue cells.

Secondary reagents were used to verify and/or help determine the effects of the three primary treatments. The effects of treatment by the three reagents (ferric chloride, hydrochloric acid, and potassium ferrocyanide) were evaluated according to the criteria listed on page 7. A ferric chloride solution was used as a source of multivalent cations and because it is used in a number of histochemical stains (18-20). Calcium and uranyl acetate solutions were also used as sources of multivalent cations, primarily to verify effects observed with the ferric ion. A ferric chloride solution did not separate the cells in canal tissue. Hydrochloric acid separated canal tissue cells but insignificantly compared with potassium ferrocyanide. The pH of the acid was always identical to the ferric chloride solution (pH 1.85) so that the effects of the hydrogen and ferric ions could be compared. Preliminary studies showed that potassium ferrocyanide was able to separate canal tissue cells without mechanical action over a wide pH range. The effect was greater than that produced by either sodium hexametaphosphate or the disodium salt of ethylenediaminetetraacetic acid. The tissue was also treated with calcium ferrocyanide and potassium chloride to help characterize the potassium ferrocyanide effect. Analytical-grade reagents were used without further purification, and salt solutions were prepared in distilled water. Salt concentrations were 0.1M except for uranyl acetate where solubility data required a 0.01M solution. The 0.1M value was selected from published histochemical studies (18-20) and the fact that Davidson (21) showed that 0.1M salt solutions exchanged better than 0.01M but similarly to 0.5M. Although ionic strength was not controlled, it was considered indirectly.

Chemical treatment of canal tissue for microscopic and cell separation evaluation was as follows. Approximately 30 canal complexes were soaked in 18-20 ml. of a filtered solution of the reagent for a predetermined period. Tissue residues

were washed, with one rinse, by soaking in water for a minimum of 4 hours. All treatments were given at 25 ± 1°C. without mechanical agitation.

Treatment of the canal complex prior to chemical analyses was as follows. The tissue was soaked in one of the reagents [ferric chloride, hydrochloric acid, or potassium ferrocyanide (pH 6.8)] for 36 hours. Tissue residues and treating liquors were separated by filtration and were dialyzed until no free ions were detected. The presence of the ferric cation was detected by using acidified potassium ferrocyanide (and vice versa) and the chloride ion with silver nitrate (22). The tissue residue and the treating and washing liquor extracts of the potassium ferrocyanide treatment were analyzed separately. In the acid and ferric chloride treatments, these two extracts were combined prior to analyses. Analytical procedures are described in Appendix III.

CELL SEPARATION AND READHESION

Qualitative estimates of the degree of cell separation and cell readhesion were obtained using the following procedures. The canal complexes were transferred from the water wash to a glass microscope slide by gently grasping them at one end with fine-nosed forceps. Water was added one drop at a time to the tissue before a cover slip was applied in the direction of the canal axis. It was essential that the amount of water be just sufficient to spread over the area covered by the cover slip, because too much water prevented surface tension forces from operating and qualitatively squashing the tissue. Although the procedure was qualitative and dependent on a number of uncontrollable variables, when applied carefully, it accurately reflected the effect of each chemical treatment on cell separation. In situations where the canal cells were not separated, 100 and/or 500-g. weights were placed on the cover slip for 30 seconds. If no separation was observed, the effect of the chemical treatment on intercellular adhesion was assumed to be

negligible. In all evaluations, the procedure was repeated at least four times and the average degree of cell separation noted. Unless the tissue was very fragile, two to four canal complexes were put under each cover slip. The degree of cell separation was recorded on 5 by 7-inch photomicrographs (magnification 34X).

Two procedures were used to investigate the ability of multivalent cations to readhere separated cells. The methods were qualitative and required suitable controls. The effect of adding ferric chloride crystals to water containing chemically and/or mechanically separated canal cells was observed directly with the light microscope. Strands of canal complex cells and/or intact canal tissue were bonded together (when in contact) by a ferric chloride or calcium acetate solution. The tissue was placed in water on a microscope slide, a cover glass applied, and a 50-g. weight added to ensure contact between adjacent cells. After removal of the cover slip under water, the degree of bonding was estimated.

LIGHT MICROSCOPY

Bright-field, phase-contrast, and polarized-light microscopy were used to evaluate tissue structure and chemistry. Cross, longitudinal, and tangential sections of the canals in both untreated wood and the holocellulose were prepared and observed. The sections were stained with phloroglucinol to detect lignin and Sudan IV to detect pitch (23). The isolated canal tissue was examined with respect to general structure, the degree of cell separation obtained with selected chemical treatments, cell wall pitting, and the extent of fiber and ray-cell contamination. Sections of the isolated tissue were used to determine the effect of the isolation procedure on structure and to help locate features visible under the electron microscope. The isolated tissue was stained with Sudan IV and Graff "C" stain to detect

pitch, and with potassium iodide-iodine to detect starch (23). Experimental procedures are described in Appendix IV.

ELECTRON MICROSCOPY

Electron microscopy constituted the major portion of the experimental work. The effect of chemical treatments on the middle lamella, cell wall, and overall tissue structure was evaluated by a variety of electron microscopic procedures using surface replicas, stained and unstained ultrathin sections, and shadowed ultrathin sections. The different methods of preparing tissue for observation with the electron microscope were selected for the following reasons:

1. To verify the existence of new structural features.
2. To identify structural artifacts (Appendix X).
3. To enable both the external (surface replicas) and internal (sections) structure of the canal complex to be observed.

The isolated canal tissue was suitable for both surface replication and ultrathin sectioning. The replicated surface was the canal complex-fiber interface. The tissue had a distinct axis, so that any structures observed could be related to wood fibers, the mature xylem, and the cambium. Surface replicas, ultrathin sections, and shadowed ultrathin sections were prepared from untreated tissue and tissue treated with ferric chloride, hydrochloric acid, and potassium ferrocyanide. For each treatment, at least four canal complexes were evaluated with respect to the three electron-microscope procedures. An RCA EMU-3F transmission electron microscope equipped with high voltage fine focusing was used.

PREPARATION OF SURFACE REPLICAS

The shadow transfer replicating procedures employed by Dunning (13) were used with the following modifications. Freeze-dried canal tissue was mounted on tinfoil,

with rubber cement at either end, prior to shadowing. The foil replaced the Metricel filter and the subsequent acetic acid wash used by Dunning. Most of the original tissue was removed from the polystyrene disk when the foil was peeled away. Remaining tissue remnants were removed from the replica by soaking overnight in 72% sulfuric acid containing calcium fluoride ($\approx$ 2%).

PREPARATION OF ULTRATHIN AND SHADOWED ULTRATHIN SECTIONS

Ultrathin and shadowed ultrathin sections vary with respect to embedding media and section properties. A maraglas epoxy-resin embedding medium (25) was used in the preparation of ultrathin sections (UTS) because it is stable under the electron beam and has negligible shrinkage (27). This resulted in an UTS of embedded canal tissue which was directly observable under the electron microscope. Shadowed ultrathin sections (STS) were prepared from tissue embedded in butyl methacrylate which is unstable under the electron beam. After sectioning, the methacrylate was removed and the remaining tissue subsequently shadowed and observed.

Two types of sections were essential for this study. Ultrathin sections showed the canal tissue in essentially an unchanged state and, in this respect, served as a control for the STS. The STS verified conclusions made from the UTS, in addition to showing new structures. Unlike an epoxy resin, butyl methacrylate shrinks up to 14% during polymerization (24). This effect gently separates adjacent cell walls of both untreated and treated canal tissue. Chemical and/or mechanical cell separation and the removal of the embedding medium revealed the "internal" structure of shadowed tissue sections. Butyl methacrylate was used as both an embedding medium and as a mechanical tool to separate adjacent cell walls.

Several features concerned with preparation of the isolated tissue for embedding are noted below.

1. Although the canal tissue was not subjected to a conventional fixation step prior to embedding, the chloriting process (Appendix I) indirectly served this function.

2. The canal complex was not visible in either of the colorless embedding media. However, the tissue could be located if it was suspended in a tinfoil framework prior to embedding. Cross and longitudinal sections were readily obtained as the tissue axis within the framework was therefore known.

3. After potassium ferrocyanide treatment, the tissue was very fragile and could not be embedded intact. This problem was overcome by adding resin to the tissue before suspending in the foil framework. Maraglas and butyl methacrylate apparently acted as adhesives between separated interfaces.

4. Throughout the chemical treatments and subsequent tissue manipulation, movement of the tissue was minimized. Mechanical movement, particularly in weakened tissue, could destroy intercellular structures.

The embedding procedures and the nature of the tinfoil framework are described in Appendix V. Maraglas (25) was selected as the epoxy embedding medium because it does not contain an anhydride. Watson and Aldridge (26) suggested that a medium containing anhydride would extract cations from tissue embedded in it. A polymerized butyl methacrylate block had a hardness suitable for sectioning the canal complex. Both longitudinal and transverse ultrathin sections (0.05 μm. thick) were cut from tissue embedded in maraglas and butyl methacrylate. Sections were cut with glass knives (LKB KnifeMaker) on a Porter-Blum ultramicrotome.

After ultrathin sections were obtained, further treatment was dependent on the embedding medium. The maraglas sections were observed before and after staining with uranyl acetate (Appendix VI). The procedures used and the effectiveness of staining the canal tissue before embedding are also discussed in Appendix VI. Butyl methacrylate was removed from ultrathin tissue sections by immersing the grids

containing the sections in chloroform for at least 12 hours. Methacrylate-free sections were shadowed at an angle of 30° with palladium (13) and observed with the electron microscope.

RESULTS AND DISCUSSION

STRUCTURE OF THE CANAL COMPLEX

Determination of the canal complex structure constituted a large part of the experimental work as it was necessary to characterize the tissue before either the middle lamella or intercellular adhesion could be investigated. The purpose of this section of the thesis is to familiarize the reader with tissue structure and terminology.

ANATOMY

Although little is known about the cell wall ultrastructure, considerable information is available regarding the anatomy of resin canal tissue in a number of Pinus species. Bannan (28), in addition to reviewing the early literature, describes the canal tissue as a duct surrounded by a single layer of thin-walled epithelial cells. This is surrounded by a variable number of both living and nonliving parenchyma cells and cell layers which normally have both unthickened and unlignified walls. Engström and Back (29) and Nyrén and Back (30) showed that the surrounding parenchyma cells are of two distinct types. The layer adjacent to the epithelium consists of short, wide cells, and the outer layer consists of narrow, elongated cells. Nyrén and Back also describe the first instance where a canal complex was isolated intact from treated wood. Panshin, et al. (31) and Howard and Manwiller (32) give general accounts of canal tissue structure in Pinus species.

The anatomy of the slash pine resin canal tissue as determined for this thesis is as follows. The canal cells are unlignified, and their walls show no evidence of secondary thickening. However, in contrast to the work of others (28-31), both the epithelial and surrounding parenchyma cells have thick primary walls.

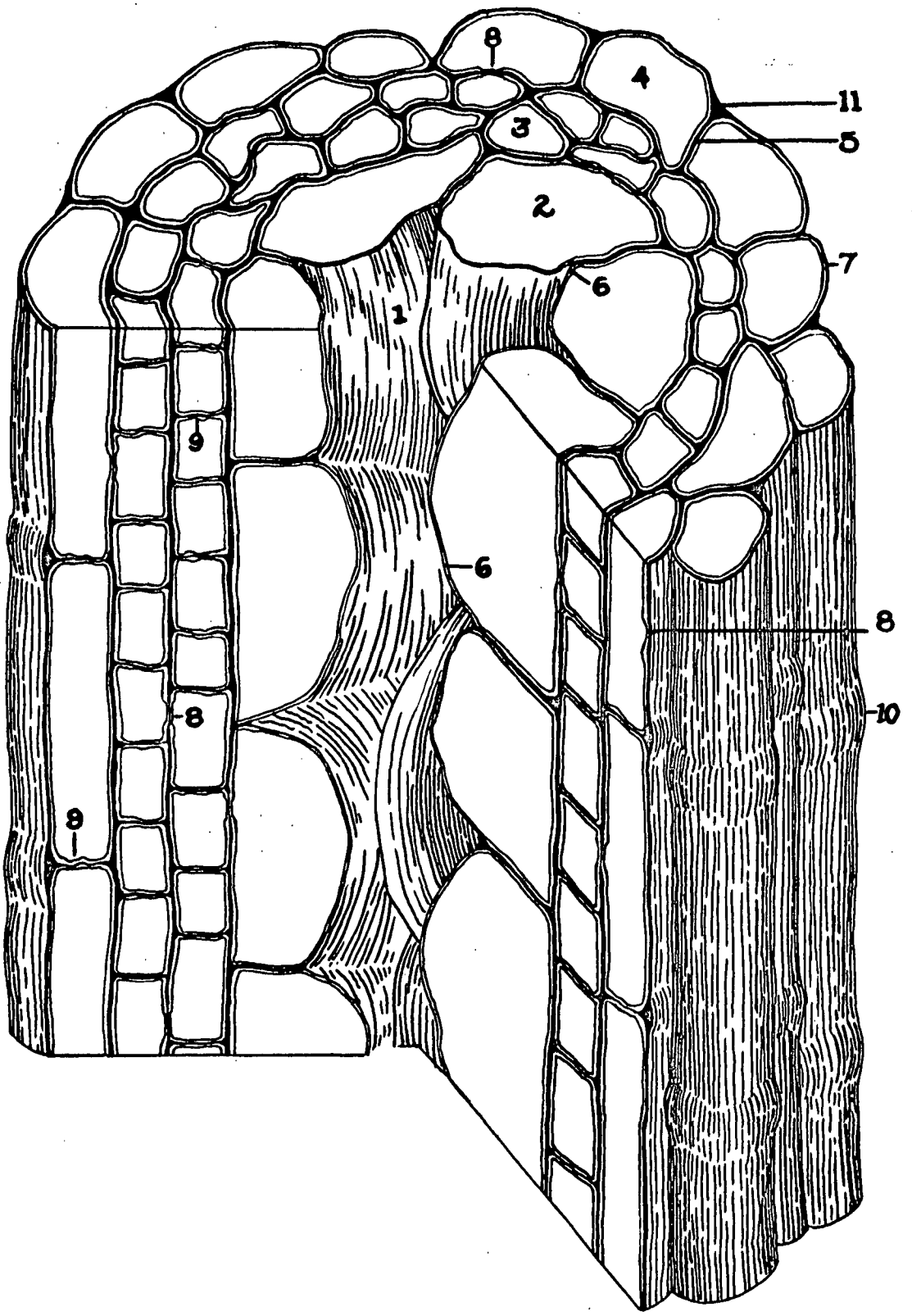
Like Pinus sylvestris (29, 30), the slash pine canal tissue consists of three types of cells. However, the epithelial cells in the slash pine canal complex vary in size and are considerably larger in both transverse and longitudinal direction than the layer of short cubelike cells, which form at least one continuous layer around the epithelium, and are called intermediate cells. Cells located adjacent to and outside the intermediate cell layer(s) may be elongated 3-4 times their diameter and are sometimes absent altogether. These are termed outer cells, and occur singly, in groups, or in layers. An outline of the general structure of the canal complex (Fig. 2) shows the relative positions and dimensions of the duct and cell types.

ULTRASTRUCTURE

The wall structure of intermediate and outer cells appears to be identical with respect to lamination, pitting, intercellular perforations and elevated cross walls. These cells apparently differ only in their dimensions and the thickness of the intercellular membranes. The thickness of the epithelial cell wall varies with the location of the wall in the canal complex. The portion of

Figure 2. The Resin Canal Complex (Drawn by Olga A. Smith)

1. Resin canal
2. Epithelial cell
3. Intermediate cell
4. Outer cell
5. Middle lamella
6. "Middle lamella" — canal lining
7. "Middle lamella" — canal complex-tracheid interface
8. Pit field
9. Location of end wall perforations
10. Elevated cross wall
11. Intercellular membrane embedded in the middle lamella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the wall adjacent to the duct is considerably thinner than the wall adjacent to the intermediate cell. The epithelial wall adjacent to the intermediate cell, as well as the intermediate cell wall, are similar in both thickness and structure. The above features are discussed in appropriate sections of this thesis and many are illustrated in Fig. 2.

CHEMISTRY OF THE CANAL COMPLEX

The effects of selected chemical treatments on the canal complex were evaluated by the cell separation and microscopic studies listed in Table I. The different pH conditions used in the potassium ferrocyanide treatments were necessary to show the effects of the reagent on cell separation. Hydrochloric acid was added to the salt solution of pH 9.4 to give pH values of 6.8 and 2.4. Various treatment periods for each reagent were run to determine when the reaction was complete. Insufficient tissue was available to determine this on a chemical composition basis, so the degree of cell separation, and changes in cell wall ultrastructure were used. After a 36-hour treatment period, no further changes in tissue structure or cell separation were observed. Consequently, all chemical-composition data were obtained from tissue treated for 36 hours and after the structural data had been evaluated.

Light microscopy showed that the canal complex is structurally identical to similar tissue in untreated wood. It is a long, hollow structure with a central cavity surrounded by 2-3 layers of structurally different cells (Fig. 2). The number of cell layers varies along the length and around the circumference of the canal. In addition, branched canals and longitudinal and radial canals are present. The canal cells were readily distinguished from the remainder of the xylem because the cell walls were not thickened and did not stain like the surrounding fibers. Sections of untreated wood stained with phloroglucinol showed that the canal tissue

TABLE I

ELECTRON MICROSCOPE AND CELL SEPARATION STUDIES MADE

Treatment	Initial pH of Treatment Liquor	Cell Separation[a]				Ultrathin Sections[a] (Transverse and Longitudinal)			Shadowed Ultrathin Sections[a] (Transverse and Longitudinal)			Surface Replicas[a]		
		6 hr.	12 hr.	36 hr.	156 hr.	6 hr.	12 hr.	36 hr.	6 hr.	12 hr.	36 hr.	6 hr.	12 hr.	36 hr.
Ferric chloride (0.1M)	1.85	+	+	+	+	+	-	+	-	-	-	+	+	+
Hydrochloric acid (≈ 0.2M)	1.85	+	+ +[c]	+ +[c]	+	+	-	-	+	-	+ +[c]	+	+	+
Potassium ferrocyanide (0.1M)	2.4	-	+ +[b] +[c]	+ +[c]	-	-	+ +[d] +[b]	+ +[d] +[b]	-	-	-	-	+ +[b]	+
	6.8	-	+ +[c]	+ +[c]	-	-	-	-	-	-	+ +[c]	-	+	+
	9.4	-	+	+	-	-	-	-	-	-	-	-	+	+

[a] Untreated control tissue examined.

[b] Ferric chloride pretreatment (0.1M, 6 hours, and pH 1.85).

[c] Ferric chloride posttreatment (0.1M, 6 hours, and pH 1.85).

[d] Uranyl acetate posttreatment (0.01M, 6 hours, and pH 4.5).

is unlignified and contains pitch. Pitch, but not starch, was detected in the canal complex. Many of the above features are illustrated in Fig. 3 and 4.

CHEMICAL ASPECTS OF INTERCELLULAR ADHESION

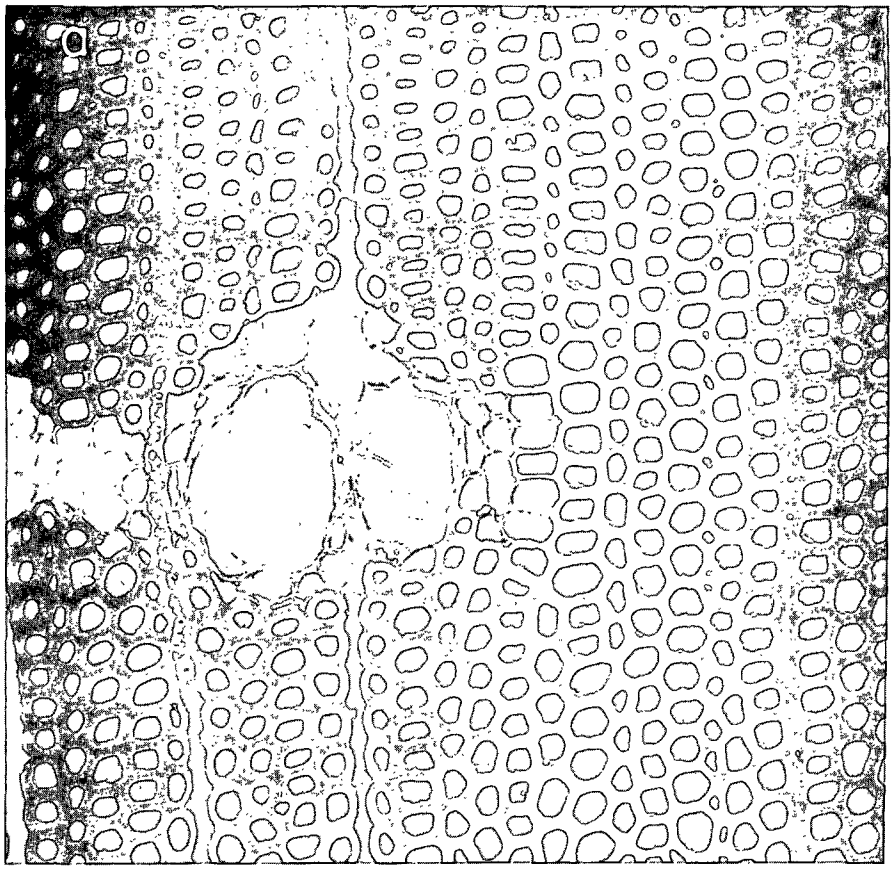
Chemical Composition

The canal complexes were isolated from three batches of holocellulose by the procedures described in Appendices I and II. Whereas the chemical composition of the holocelluloses were practically identical, the chemical composition of the three lots of canal tissue differed (Tables II and III). Variation between Canal Tissues A and B was due to their galacturonic acid and calcium content. The loss of these substances from Tissue B was traced to overheating (75°C.) during purification (Appendix II). The high mannan and glucan content and the low galactan and galacturonic acid content in Canal Tissue C was attributed to the elimination of the mild sequestering stage during purification (Appendix II). This stage was excluded in Tissue C to obtain a quantitative measure of the fibrous material it removed. The low percentage of substances accounted for in Table III was probably due to the existence of pitch and cytoplasmic materials in the canal tissue.

Figure 3. Cross Sections of Untreated Slash Pinewood Stained with Phloroglucinol. Both Photomicrographs Show the Gross Structure of the Canal Tissue and Associated Fibers. The Unstained and, Therefore, Unlignified Canal and Ray Tissue are Evident. In (b), Pitch Globules are Visible in the Ray and Canal Cells

Plate Numbers: a. 2-5
b. 2-4

Magnification: 35X


a

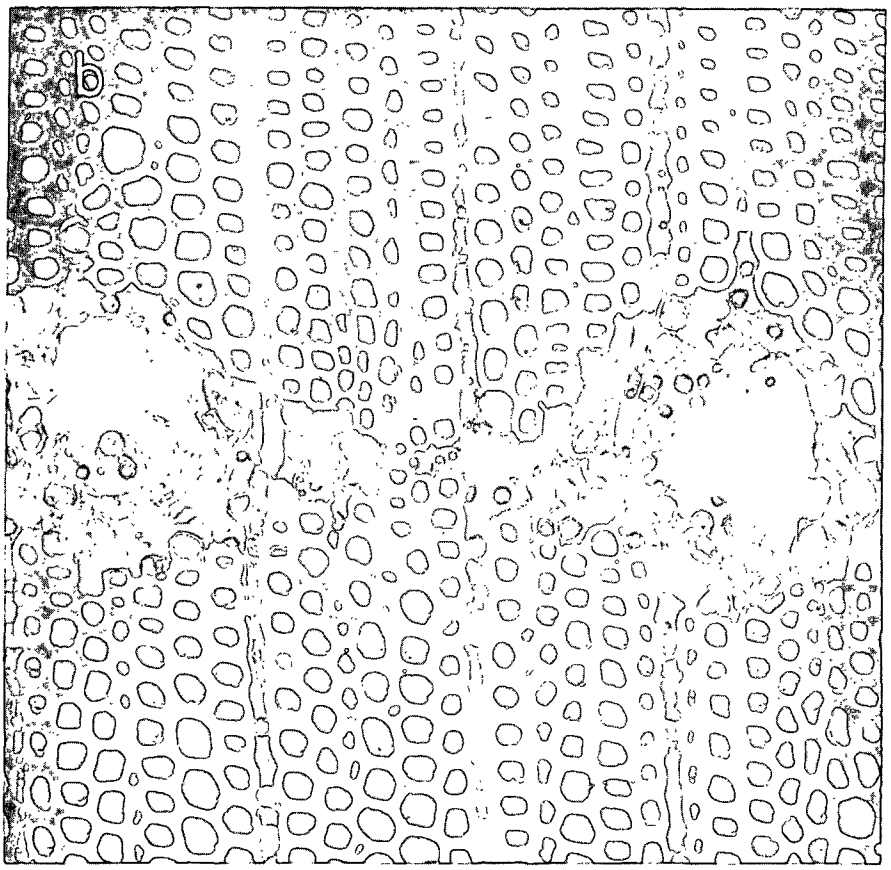

b

Figure 4. Cross Sections of Canal Complexes Embedded in Maraglas (a) and Butyl Methacrylate (b). Adjacent Cell Walls in the Methacrylate Section (b) are Separated and Distorted to a Greater Extent than in the Maraglas Section (a)

D; Canal
E: Epithelial cell
I: Intermediate cell
O: Outer cell
R: Ray cell

Plate Numbers: a. 04-19
b. 68M-659C

Magnification: a. 510X
b. 450X

TABLE II

CHEMICAL COMPOSITION OF HOLOCELLULOSE AND CANAL TISSUE[a]

	Holocellulose			Canal Tissue[b]		
	A	B	C	A	B	C
Sugars						
Rhamnan	ND	ND	ND	1.0	0.7	0.4
Araban	0.5	0.5	0.5	3.1	2.5	1.7
Xylan	4.5	4.5	4.9	6.6	6.3	6.4
Mannan	13.9	15.0	14.9	5.3	6.8	11.1
Galactan	1.7	1.2	1.4	5.6	5.7	3.1
Glucan	76.5	76.1	76.5	48.9	56.1	66.9
Cations						
Calcium	0.7	0.7	0.3	4.9	3.5	2.3
Sodium	1.5	1.4	1.2	0.1	0.2	0.1
Others[c]	0.01	0.06	0.02	0.1	0.5	0.5
Galacturonic acid	0.4	0.5	0.3	24.5	17.5	7.6
Organic nitrogen	0.02	0.02	NA	0.1	0.2	NA

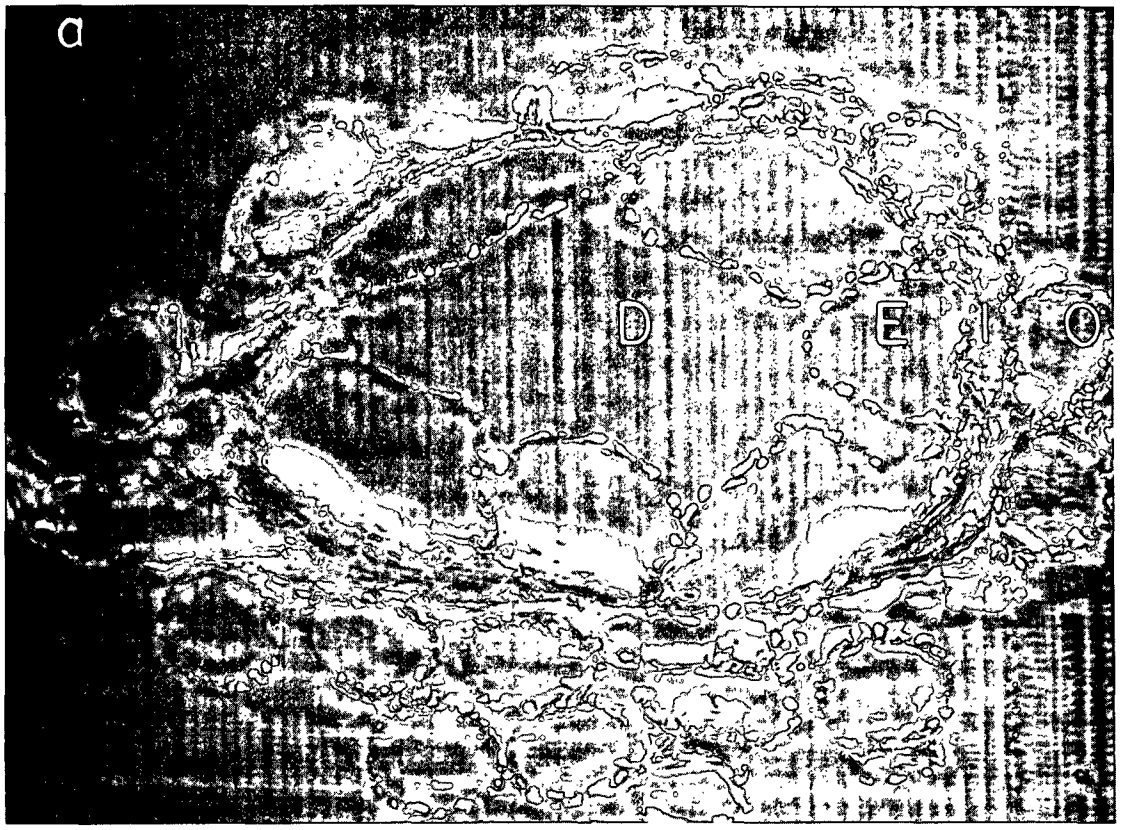
[a] Based on percentage of substances analyzed.

[b] A: Canal tissue from holocellulose A was used in the microscopic and cell separation studies.
B: Canal tissue from holocellulose B was used in the chemical composition studies.
C: Canal tissue from holocellulose C was used in the chemical composition studies. Sequestering stage eliminated during canal complex purification.

[c] See Appendix VII.

NA = Not analyzed.

ND = Not detected.


a
D
E
I
O

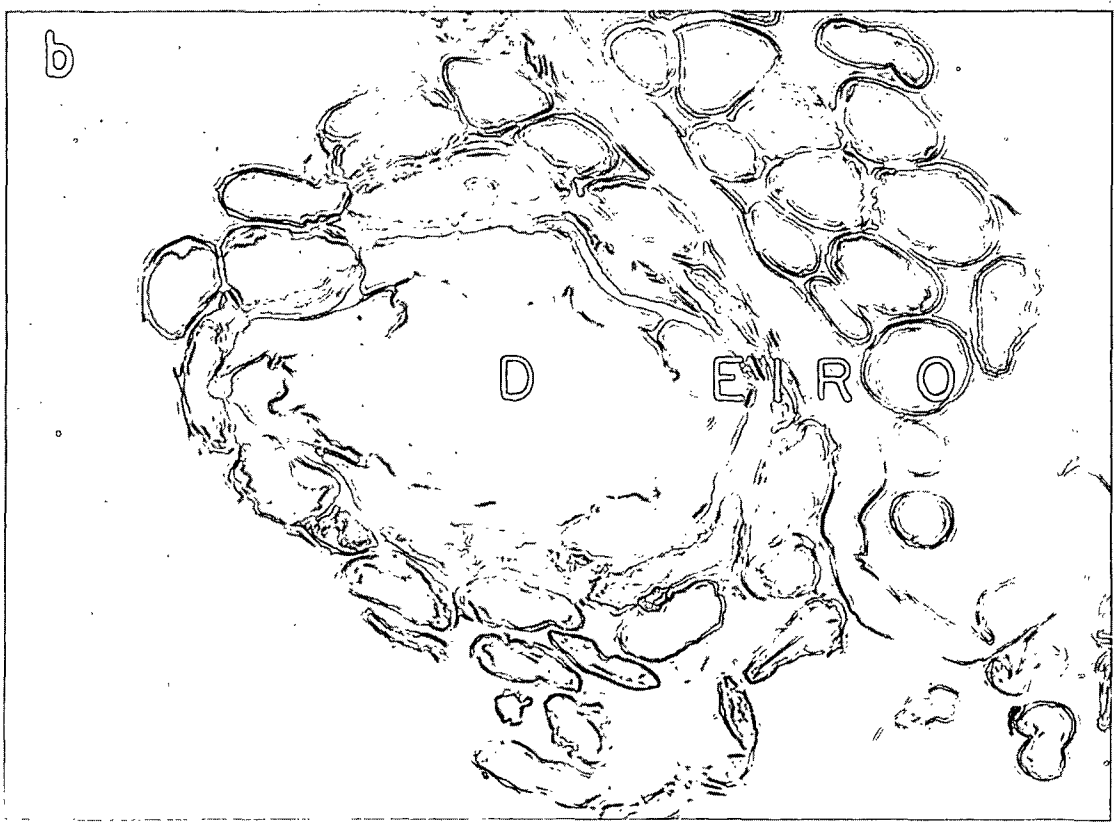

b
D
E
I
R
O

TABLE III

ANALYSIS OF HOLOCELLULOSE AND CANAL TISSUE[a]

	Holocellulose			Canal Tissue[b]		
	A	B	C	A	B	C
Uronic acids less galacturonic acid	4.2	5.1	NA	3.7	NA	NA
Methoxyl	NA	NA	NA	1.7	NA	NA
Acetyl	NA	NA	NA	ND	NA	NA
Klason lignin	ND	0.04	ND	NA	NA	NA
Percent tissue accounted for in Tables II and III	97.0	91.8	88.4	73.8	75.0	81.4

[a] Same tissue as shown in Table II. Data based on percentage of substances analyzed in Tables II and III.

[b] A: Canal tissue from holocellulose A was used in the microscopic and cell separation studies.
B: Canal tissue from holocellulose B was used in the chemical composition studies.
C: Canal tissue from holocellulose C was used in the chemical composition studies. Sequestering stage eliminated during canal complex purification.

NA = Not analyzed.

ND = Not detected.

Thompson, et al. (15) determined the chemical composition of a holocellulose sample from longleaf pine and of the canal tissue isolated from it. His data were similar to those listed in Tables II and III of this study. In both studies, canal tissue was rich in hemicelluloses and uronic acids and contained a large amount (> 20%) of noncarbohydrate material. Mannan was the only substance analyzed which decreased rather than increased when the canal tissue was compared with the respective holocellulose. In contrast to Thompson's data, starch was absent, and small amounts of rhamnan were detected in the canal complexes of slash pine.

A small sample of Canal Tissue A (Tables II and III) was extracted with a 10% aqueous, potassium hydroxide solution. The extract was neutralized with acetic acid and tested with a potassium iodide-iodine reagent (23). The resulting color was greenish-blue, similar to that described by Thompson, et al. (33) for an "amyloid"-containing potassium hydroxide extract, obtained from the holocellulose of immature jack pinewood. The solution turned brown after warming to ≈ 60°C. and deep blue on cooling to ≈ 15°C. The deep blue color of a similarly tested starch solution remained unchanged. Although insufficient material was available to purify the extract (33), the relative sugar content was determined. Data listed in Table IV show that the sugar ratios in the extract essentially agree with those of Thompson, et al. (33). In addition, the known compositions of a variety of amyloids (34-36) confirmed that the "amyloid" substances found in the canal tissue lie within the range of published values.

TABLE IV

CARBOHYDRATE COMPOSITION OF EXTRACTS CONTAINING "AMYLOID"

	Rhamnan	Araban	Xylan	Mannan	Galactan	Glucan
10% KOH extract of canal tissue A[a]	4.4	8.2	34.0	6.7	17.3	29.4
10% KOH extract of immature jack pine[b]	ND	3.1	28.0	4.9	24.0	40.0
Aqueous extract of tamarind seed[b]	ND	1.3	34.2	ND	16.9	47.6

[a]Canal tissue A: See Tables II and III.

[b]Data published by Thompson, et al. (33).

ND = Not detected.

Cell Separation

Ferric chloride, hydrochloric acid, and potassium ferrocyanide were used for the treatments because they produced different degrees of cell separation (Fig. 5).

Figure 5. The Effect of Selected Chemical Treatments on the Canal Complex

a. An untreated canal complex essentially void of ray cells (atypical)
b. An untreated canal complex associated with both ray and radial canal tissue. Ray-cell contamination is typical

O: Outer cells
R: Ray cells
T: Radial canal tissue

c. A canal complex which was treated in hydrochloric acid and subsequently washed in water
d. A canal complex which was treated in potassium ferrocyanide at pH 6.8 and subsequently washed in water

Plate Numbers: a. 68M-636Q
b. 68M-636T
c. 68M-636AZ
d. 68M-636AT

Magnification: 33X

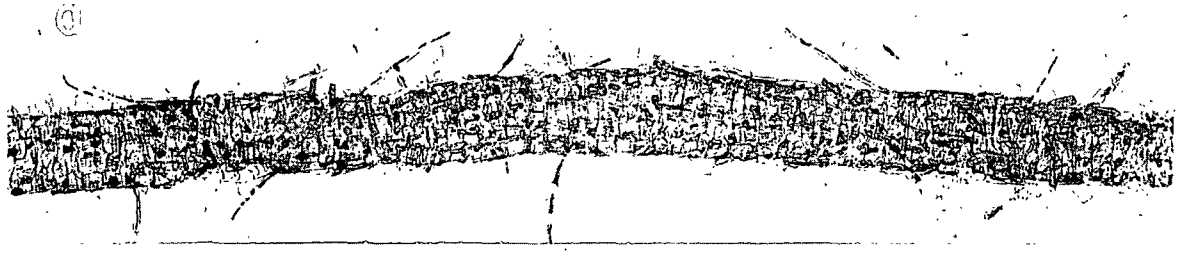
Additional reagents were used to help characterize the effects observed. Canal complex cells separated according to the following series:

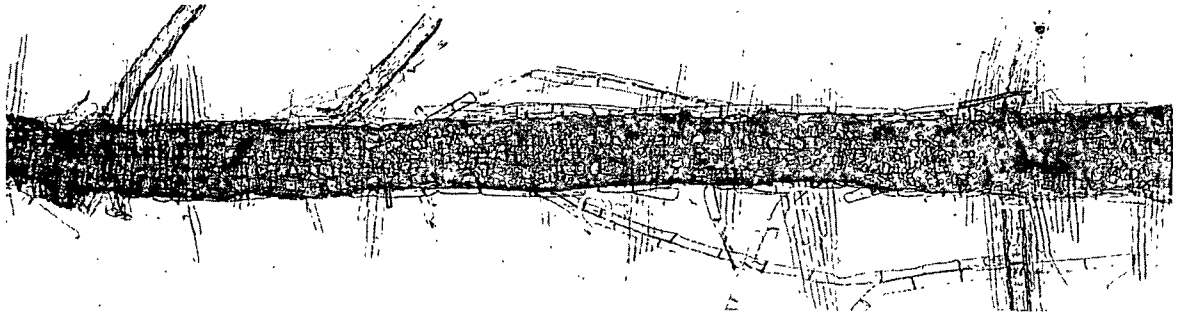
untreated ≅ ferric chloride (pH 1.85), uranyl acetate (pH 4.5) and calcium acetate (pH 4.5) ≅ calcium ferrocyanide (pH 2.4, 6.8) ≅ potassium hydroxide (10%)

< hydrochloric acid (pH 1.85) < potassium chloride (pH 2.4, 6.8) ≅ potassium ferrocyanide (pH 2.4) < potassium ferrocyanide (pH 9.4) ≤ potassium ferrocyanide (pH 6.8).

The test employed to estimate the degree of cell separation (page 13) was not sufficiently sensitive to distinguish between the pH 9.4 and 6.8 potassium ferrocyanide treatments. Tissue treated at pH 6.8 appeared to be slightly weaker, so it was used in the chemical composition and most of the structural studies. Treatments with potassium chloride and calcium ferrocyanide confirmed that the ability of potassium ferrocyanide to separate canal cells was related to both the potassium and ferrocyanide ions.

Cell separation occurred only after washing the treated tissue in water. The effect of washing was observed in potassium chloride, potassium ferrocyanide, and





to a lesser extent in acid. This indicates that cell separation after washing was caused by either extraction of material that was insoluble prior to treatment, or a charge effect. These alternatives were studied by comparing the degree of cell separation obtained from unwashed, water-washed, and potassium chloride-washed tissue treated with potassium ferrocyanide. Significant separation occurred only in the water-washed material. Because chemical composition data (Table V) showed that the washing water extracted only small amounts of sugars and galacturonic acid from the tissue residue, cell separation was attributed to a charge effect as discussed later.

TABLE V

CHEMICAL ANALYSES OF TISSUE TREATED WITH POTASSIUM FERROCYANIDE[a]

	Rhamnan	Araban	Xylan	Mannan	Galactan	Glucan	Galacturonic Acid	Calcium	Potassium
Tissue residue	1.0	2.0	9.0	11.1	5.8	97.8	5.7	0.4	ND
Liquor extract	0.4	0.6	0.2	ND	0.8	1.2	13.1	**[b]	**[b]
Washing extract	0.2	0.5	0.4	0.4	0.8	1.0	2.2	**[b]	**[b]

[a] Based on 100 units of glucose (residue plus extracts).

[b] See Appendix VIII.

ND = Not detected.

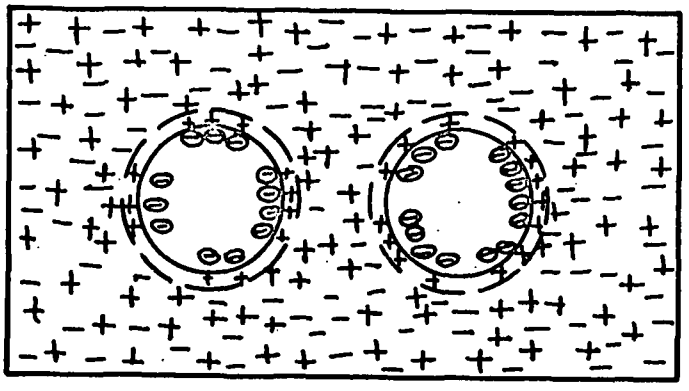
Data in Table V were calculated on the assumption that no glucose was lost from the extracts. Although this assumption was not completely valid, it did allow extracts and treatments to be compared. While the hemicellulose content in the extracts was considered negligible, the calcium and galacturonic acid data need to be explained. The data show that some calcium was extracted during the washing treatment. Due to

the insolubility of calcium pectate, most of the calcium extracted must have been complexed with residual ferrocyanide. This is verified by the small quantity of galacturonic acid extracted during washing. The extracts as well as their calcium and potassium contents are discussed further in Appendices VIII and IX.

This study showed that during the potassium ferrocyanide treatment, calcium was extracted and replaced with potassium. The water wash extracted potassium from the residue and electrical double layers which consisted of the potassium and ionized acidic groups expanded (12). During expansion of the double layers, repulsion effects developed between adjacent wall elements and caused cell separation by tissue swelling. The ultrastructure studies (page 48) indicated that electrical double layers existed over at least the fibrillar elements of the primary wall and the middle lamella.

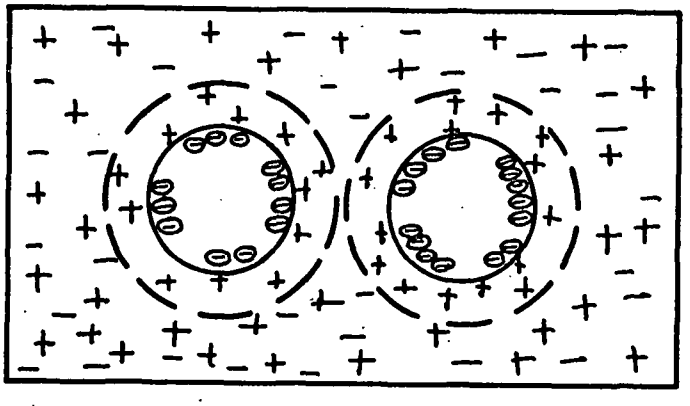
The highly simplified diagrams in Fig. 6 show how the surfaces of two adjacent wall elements may be forced apart by expansion of their electrical double layers. Although the magnitude of the charge in the surfaces is not changed, the effective charge extends into the surrounding medium, via the diffuse part of the double layer, as the salt concentration decreases. When the electrolyte of the external medium becomes very dilute (Fig. 6c), the double layers on adjacent wall elements expand to the stage where they interact. This causes adjacent wall elements, and eventually adjacent cell walls, to be forced apart by repulsion. This theory is supported by the fact that canal cells are not separated until after the initial washing water is replaced. Additional evidence which supports this conclusion is obtained from the chemical analyses. Potassium was not detected in significant amounts in the washed, potassium ferrocyanide treated tissue. In addition, the washing effect was partially reproducible when the tissue was treated with potassium ferrocyanide, washed, soaked in calcium acetate to restrengthen, treated again, and washed.

(a)



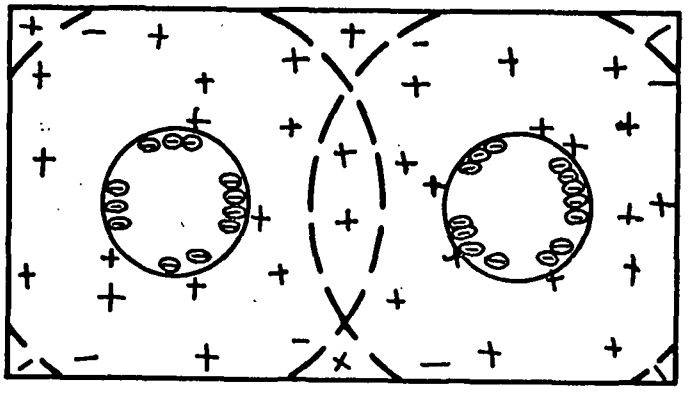
Electrical double layer collapsed due to a high potassium ferrocyanide concentration. The circled structures, ⊖ , represent ionized acidic groups in the surface of a structural element in the cell wall.

(b)



Electrical double layer expanded slightly with decrease in concentration.

(c)



Electrical double layer expanded to the extent that adjacent wall elements are separated by repulsion.

Figure 6. A Highly Simplified Illustration of the Effect of Electrical Double Layer Expansion on Cell Separation

This essentially verified that a charge effect, rather than extraction of non-cationic bonding substances, was involved in the washing phenomenon.

Ginzberg (6) considered the washing effect using ethylenediaminetetraacetic acid (EDTA) and root tips of Alaska pea seedlings. Root tips were soaked in a series of EDTA concentrations and either examined immediately or after rinsing in water. When the tissue was examined immediately after treatment, cell separation decreased as the EDTA concentration was increased. After the treated tissue was soaked in water, the degree of separation was independent of the EDTA concentration. To confirm that ionic strength was controlling cell separation, Ginzberg studied the effect of adding various concentrations of potassium chloride and lithium chloride to a 0.1M EDTA solution. The degree of separation decreased when the ionic strength was increased. Although the effect of ionic strength is quite apparent from these experiments, Ginzberg's conclusion that these data "point to the existence of a charge either in the intercellular cement or on the cell wall surface" is obscure. Ginzberg did not discuss the nature of the charge or whether it exists prior to treatment, during treatment, or when the treated tissue is soaked in water. Nor did he discuss whether the charge resists or promotes cell separation. These studies were apparently omitted because they were not directly associated with the emphasis of his study.

Ginzberg's experimental approach was not suitable to investigate the existence and nature of a charge effect because he did not consider the effects of treatment on tissue structure and chemistry. Therefore, a more reasonable explanation of Ginzberg's data is proposed. When multivalent cations are removed from the tissue by chelating agents, most of the acidic substances are solubilized (9) and are extracted. In addition, Taylor and Wain (9) showed that the ionic strength of the external medium controls both the quality and quantity of substances extracted from

the tissue. Substances retained in the tissue probably caused the variation in cell separation noted by Ginzberg. At the ionic strengths employed by Ginzberg ($\geq$ 0.05M), a charge associated with ionized acidic groups on the surfaces of any structures within the cell walls or the middle lamella is unlikely because concentrations were sufficiently high to collapse any electrical double layers. Chemical composition analyses at various ionic strengths are necessary before an adequate explanation of the effect of ionic strength on cell separation (in tissues treated but not washed) can be determined.

The canal complex was stained a deep blue when a ferric-ferrocyanide complex (19) was produced on the surface and within the canal cells. This treatment also caused the canal cells to separate so that the shape and relative size of the different cell types were readily visible (Fig. 7).

Figure 7. A Canal Complex Before (a) and After (b) a Cover Glass was Applied

E: Epithelial cells
I: Intermediate cells
O: Outer cells
R: Ray cells

Plate Numbers: a. 68M-636I
b. 68M-636F

Magnification: 34X

Tissue Treatment: Soaked in ferric chloride for 6 hours, washed, soaked in acidified potassium ferrocyanide for 12 hours, and washed again

Chemical Treatment

The effects of selected chemical treatments on the canal complex were evaluated by determining the amounts of galacturonic acid, sugars, cations, and organic nitrogen in the washed tissue residues. Galacturonic acid and calcium were selected as a measure of the uronic acids and multivalent cations. These substances constituted a large portion of the canal complex (Table II) and are apparently associated with intercellular adhesion (1-9). Ginzberg (6) concluded that protein was important to intercellular adhesion; therefore, organic nitrogen was included in the analyses. However, because of the large quantity of tissue required, the nitrogen analysis was confined to the untreated tissue and that treated with potassium ferrocyanide. Sugars were also included in the analyses because they are associated with pectic substances (37) and because their role in intercellular adhesion is unknown.

The chemical composition study was designed to determine which substances are important to intercellular adhesion in the canal complex. This was achieved by relating the composition of treated tissue to the degree of cell separation. Composition data are listed in Table VI and are based on the analytical results discussed in Appendices VIII and IX. These data show that:

1. The ferric cation replaced almost all other cations in the tissue (Appendix VIII), but the treatment did not cause the cells to separate. The degree of cation exchange was greater than that obtained with either the acid or potassium ferrocyanide treatments and indicated that no barriers to exchange existed.

2. The degree of cell separation in tissue treated with hydrochloric acid and subsequently washed (Fig. 5) was primarily attributed to the extraction of multivalent cations and most of the galacturonic acid. According to Stoddart, et al. (37), the large amounts of araban and probably galactan extracted was due to their association with galacturonic acid.

3. Although the composition of tissue treated with potassium ferrocyanide was essentially identical to tissue treated with acid, the degree of cell separation was much greater in the former. The only apparent difference in composition was a lower galacturonic acid content. This was significant as the multivalent cation contents were similar.

TABLE VI

CHEMICAL COMPOSITION DATA[a]

	Treatment (Canal Tissue B[b])				Treatment (Canal Tissue C[b])		
	Untreated Tissue	Ferric Chloride (0.1M pH 1.9)	Hydrochloric Acid (pH 1.9)	Potassium Ferrocyanide (0.1M pH 6.8)	Untreated Tissue	Hydrochloric Acid (pH 1.9)	Potassium Ferrocyanide (0.1M pH 6.8)
Rhamnan	1.2	1.4	0.6	1.0	0.6	0.3	0.4
Araban	4.5	3.4	1.5	2.0	2.6	1.1	0.9
Xylan	11.2	9.6	8.8	9.0	9.6	8.1	7.2
Mannan	12.1	10.1	11.5	11.1	16.6	15.3	15.0
Galactan	10.2	8.5	6.3	5.8	4.6	5.1	2.9
Glucan	100.0	96.6	98.5	97.8	100.0	96.8	96.8
Galacturonic acid	31.2	28.7	8.8	5.7	11.4	4.6	3.2
Organic nitrogen	0.3	NA	NA	0.2	NA	NA	NA
Calcium	6.2	0.1	0.5	0.4	3.4	0.2	0.2
Iron	0.4	9.7	0.1	0.1	0.04	0.02	0.06

[a]Based on total glucose content (residue plus extract equals 100 units).

[b]See Table II.

NA = Not analyzed.

4. The tissue treated with acid and potassium ferrocyanide contained an excess of galacturonic acid relative to the multivalent cations. With reference to Tissue B (Table VI), the molecular ratios of calcium to galacturonic acid were 1:1.5 (untreated tissue), 1:4.7 (acid treatment), and 1:3.5 (potassium ferrocyanide treatment). Similar trends were obtained from the data on Tissue C (1:0.8, 1:6.6, and 1:4.4). The presence of large amounts of residual galacturonic acid was attributed to the method of treatment. The tissue was soaked in a reagent without mechanical action, and hence, diffusion was the only way in which substances were removed.

5. The difference in the amounts of nitrogen detected in the untreated tissue and that treated by potassium ferrocyanide was very small. Hence, substances containing nitrogen apparently were not associated with intercellular adhesion in the canal complex. The canal tissue was a poor material with which to study the role of organic nitrogen on intercellular adhesion because the chloriting procedure removed most of the cell wall proteins (38), and the cells retained some of their cytoplasmic contents. In addition, the effect of removing cations masked any effect due to nitrogen-containing substances.

The similar chemical compositions but different degrees of cell separation in the acid and potassium ferrocyanide-treated tissue were explained in terms of a charge effect (page 30ff). Chowdhury and Neale (39), working with carboxymethyl cellulose, showed that neutral salts such as potassium chloride depressed the pK_m and suppressed electrostatic interactions between carboxyl groups. The pK_m was defined as that pH where the carboxyls were 50% dissociated. Electrostatic interactions were described as the ability of an ionized group to hinder the dissociation of surrounding carboxyls. Hydrogen bonding between carboxyls in close proximity may be a simplified interpretation of these interactions (43). Similar trends were obtained by other workers using polymethacrylic acid (40, 41) and carboxymethyl cellulose (42). The conclusions of these workers explain the effects observed with the canal complex in this study. Cell separation in the tissue treated with potassium ferrocyanide (pH 2.4) and potassium chloride (pH 2.4) resulted only after the tissue had been soaked in water, and was attributed to a charge effect. As a result of a decrease in the pK_m, the effective pH of these reagents, in terms of carboxyl ionization, was considerably greater than 2.4. Hence, the degree of ionization in

the unwashed tissue was greater than in the acid-treated tissue. When the tissue residues were soaked in water, potassium was extracted, the electrical double layers were expanded, and the cells were separated by repulsion and subsequent tissue swelling. The degree of separation in tissue treated with potassium ferrocyanide at pH 6.8 was greater than at pH 2.4 because ionization was essentially complete and, therefore, the charge effect was larger. The acid-treated tissue was virtually unaffected by washing, due to weak ionization. Ionization probably only increased slightly when the tissue residue was soaked in water (pH $\approx$ 6) and accounted for the small increase in cell separation. Electrostatic interactions between carboxyls resisted ionization (39, 43) and therefore tissue swelling. Hydrogen bonding between the carboxyls of different molecules also explained the higher galacturonic acid content in the acid-treated tissue (Table VI). The relative sizes of the hydronium ion and the solvated potassium ion must have affected the thickness of the electrical double layers (45) in canal tissue which was washed after treatment in acid or potassium ferrocyanide. However, the effect of ion size and solvation was probably small compared with the electrostatic interactions which resisted ionization when acid-treated tissue was soaked in water (44).

The role of multivalent cations in the primary cell wall and/or the middle lamella of the canal complex is to stabilize and prevent solution of the acidic substances. Removal of the cations and most of the acidic substances does not cause substantial cell separation unless the tissue is soaked in an aqueous medium of approximately zero ionic strength or is subjected to mechanical agitation. Therefore, the cation-free primary wall and/or middle lamella must contain bonding or structural features which resist cell separation until it is subjected to swelling or mechanical stress. Of the tissue residues studied, the washed, acid-treated tissue most nearly represents the effect of removing multivalent cations.

The conclusions reached in this study with the canal complex contradict previous concepts regarding intercellular adhesion. The canal complex was isolated from a wood holocellulose, so results obtained with it cannot be directly related to plant tissues. However, it is evident that the conclusions of Ginzberg (6) and Letham (7) are essentially meaningless as their cell separation data were not related to chemical composition.

CHEMICAL ASPECTS OF CELL WALL ULTRASTRUCTURE

Cell Separation

Tissue distortion was not observed in ultrathin sections of the canal complex embedded in maraglas epoxy resin (Fig. 4). A cross-sectional view of the untreated canal complex in Fig. 8 shows the densely stained middle lamella and the thick laminated walls. However, the boundaries of the middle lamella are not traceable, due to variable contrast. The effects of treating the canal complex with hydrochloric acid or potassium ferrocyanide and subsequently soaking it in water are shown in Fig. 9. Cells and cell-wall lamellae are separated, and the densely stained middle lamella has disappeared. The different degrees of cell separation were discussed in the preceding section and were attributed to a charge effect developed during washing. Although cells of the acid-treated tissue were separated slightly during

Figure 8. Stained, Ultrathin Cross Section of an Untreated Canal Complex

Ar: Artifact (Appendix X)
C: Cytoplasmic remnant
D: Canal
E: Epithelial cell
I: Intermediate cell
ML: Middle lamella

Plate Number: 5894F

Magnification: 7900X


I
E
D
ML
E
Af
Ar
C
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I
2um

washing, the effect was small. Figure 9a represents most closely the effect of removing the multivalent cations and most of the acidic substances (Table VI). The charge effect which developed when the tissue treated with potassium ferrocyanide was washed is apparent when Fig. 9a and b are compared. Examination of Fig. 9b shows that the wall thickness of the central cells is similar to the remainder in this photo.

Although the stained ultrathin sections furnished considerable information, few conclusions were reached with them. This was due to their dependence on contrast by staining and to the presence of the embedding medium which prevented structures not in the section surface from being stained. For these reasons, shadowed ultrathin sections were used to confirm features predicted from studying the stained sections and also to identify structural aspects of the middle lamella.

Figure 10 shows the fine structural detail which resulted from removing the embedding media before shadowing the sections. When the shadowed section of tissue treated with hydrochloric acid and washed in water (Fig. 10a) is compared with the

Figure 9. Stained, Ultrathin Cross Sections of Treated Canal Complexes Showing the Different Degrees of Cell Wall Separation After Soaking in Water.

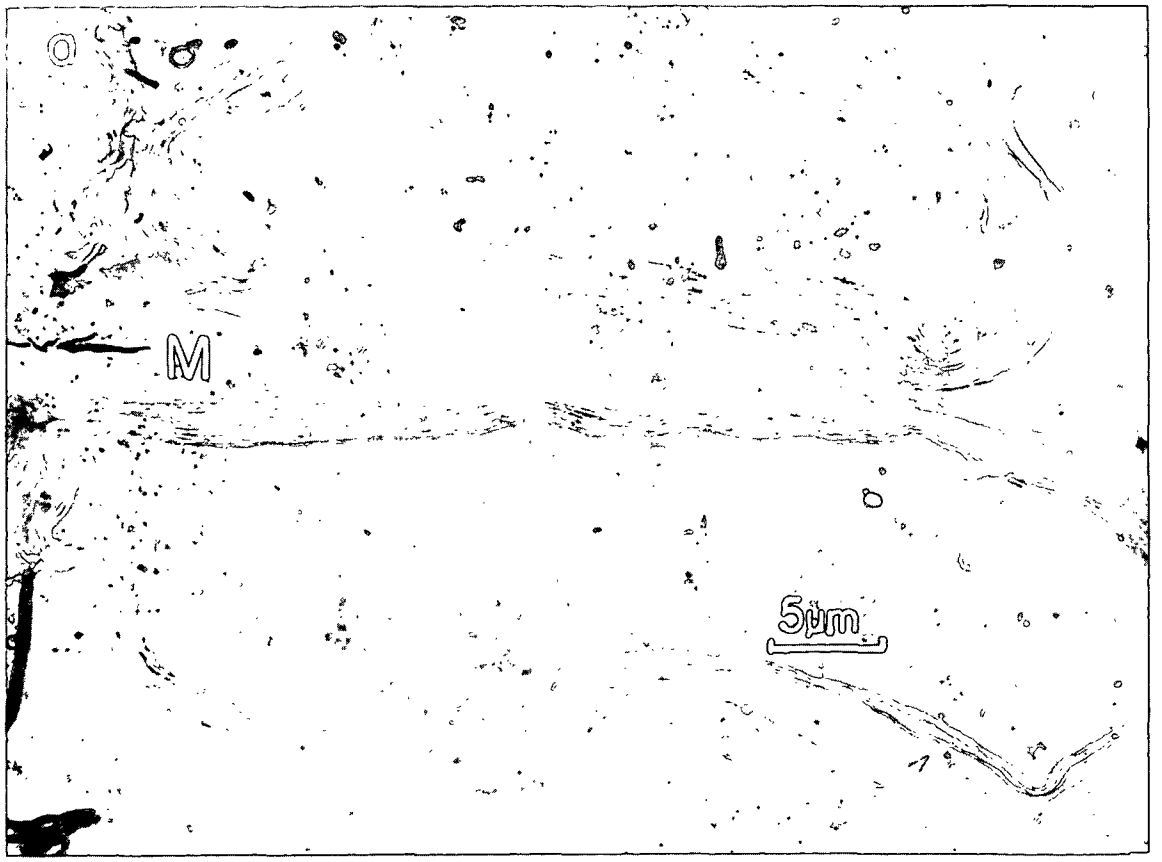
M: Intercellular membrane

Plate Numbers: a. 6029F
b. 6109F

Magnification: 3000X

Tissue Treatment: a. Soaked in hydrochloric acid for 6 hours and washed.

b. Soaked in potassium ferrocyanide at pH 2.4 for 36 hours; washed, soaked in uranyl acetate for 6 hours; and washed again.


a
M
5μm

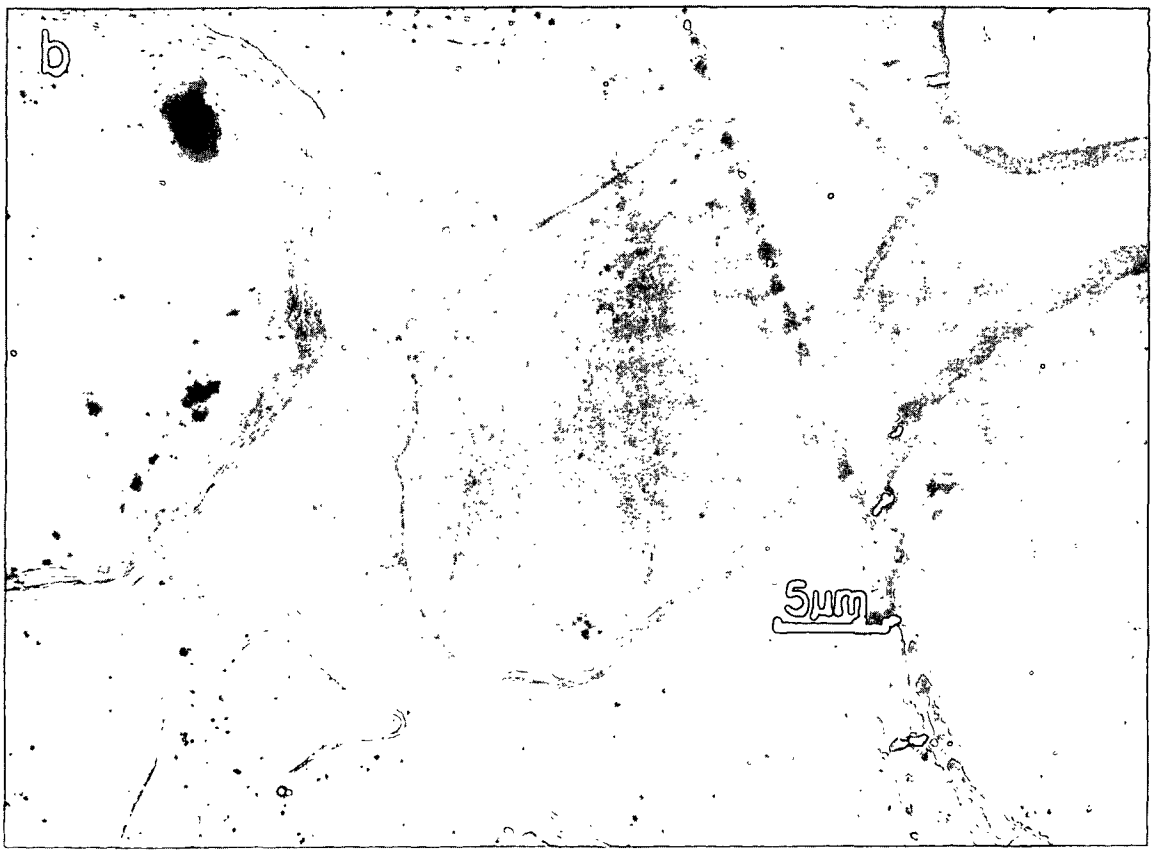

b
5μm

Figure 10. Shadowed, Ultrathin Cross Sections of Treated Canal Complexes Showing the Different Degrees of Cell Wall Separation After Soaking in Water

C: Cytoplasmic remnant*
E: Epithelial cell
F: Intercell-wall fibrils
I: Intermediate cell
L: Cell lumen
M: Intercellular membrane
O: Outer cell
PF: Pit field

Plate Numbers: a. 6752AF
b. 6765AF

Magnification: 2600X

Tissue Treatment: a. Soaked in hydrochloric acid for 36 hours and washed.

b. Soaked in potassium ferrocyanide at pH 6.8 for 36 hours and washed.

*Cytoplasmic remnants are also visible in maraglas embedded tissue (Fig. 8), and are apparently not closely associated with the cell walls of the resin canal tissue isolated from a chlorite holocellulose. Examination of electron micrographs of maraglas embedded tissue shows that the highly coiled appearance of the cytoplasmic remnants is attributable to chemical treatment rather than polymerization of the butyl methacrylate embedding medium.


a
M
Q
F
5μm
I
PF
E


b
L
L
M
F
M
5μm
F
L
L

stained section in Fig. 9a, little swelling or distortion of the tissue is evident. This indicates that cell separation and wall thickness are increased only slightly by polymerization of the butyl methacrylate embedding medium. Figures 8, 9, and 10 show that while multivalent cations were removed from throughout the cell wall by the acid treatment, the greatest majority were taken from the middle lamella. Because the washed, acid and potassium ferrocyanide-treated tissues have similar compositions (Table VI), differences in Fig. 10a and b were attributed to the washing effect and to shrinkage of butyl methacrylate during polymerization. The tissue treated with potassium ferrocyanide was weakened and then swollen by the charge effect developed during washing, and, hence, shrinkage during polymerization distorted (24) and subsequently separated adjacent cell walls to a greater degree than in the acid-treated tissue. Figures 9b and 10b are not comparable, as different pH conditions were employed during treatment.

Complete cell separation in canal tissue treated with potassium ferrocyanide and subsequently washed, was apparently prevented by intercellular membranes and intercell-wall fibrils (Fig. 10). In addition, separation in the washed, acid-treated tissue was resisted by the previously described hydrogen bonding effects (page 40). In accordance with Fig. 10 and Table VI, the residual hemicellulose and acidic substances must be closely associated with the intercell-wall cellulosic fibrils. These substances may form a network which surrounds or connects adjacent fibrils and, hence, have a role in intercellular adhesion. The rupture of such connections, as a result of the washing effect and methacrylate shrinkage, would explain the open fibril network in potassium ferrocyanide-treated tissue. Because the charge effect was small in the acid-treated canal complexes, the polymeric interfibrillar connections were not ruptured.

Remnants of a pit field are visible in Fig. 10a. That portion of the primary cell wall associated with the field is thin and indented in relation to the remainder. The structure is called a pit field rather than a pit because the cell walls of the canal complex are without secondary thickening (3). Surface views of pit fields in untreated tissue and tissue treated with ferric chloride are shown in Fig. 11. Because these structures, or remnants of them, were not identified in surface replicas of washed, acid or potassium ferrocyanide-treated tissue, it was concluded that the middle lamella [fiber-canal complex interface (Fig. 2)] was being observed. The absence of middle lamella substances in the pit field remnant in Fig. 10 supported this view. The untreated canal complex (Fig. 11) had a skin-like layer covering the surface which was absent in ferric chloride-treated tissue. This layer may well be the nitrogen-containing "skin substance" described by Ludtke and Lerch (38).

Noncellulosic Fibrils

Surface replicas of canal complexes which were treated in hydrochloric acid and potassium ferrocyanide (pH 2.4, 6.8) and then washed, showed decomposition and eventual disappearance of fibril-like structures. The electron micrographs in Fig. 12 show an untreated canal complex with fibrillar material embedded in an amorphous matrix (a) as well as uplifted fibrillar networks on a partially treated surface from which much of the amorphous substances have been removed (b). Figure 13 shows a partially treated surface (a) and a surface representative of extended treatment (36 hours) (b). The uplifted, fibrillar networks were not detected in the canal complex after extended treatment. Potassium ferrocyanide treatment at pH 6.8 was more effective than at pH 2.4. At pH 6.8, treatment was nearly complete after only 12 hours, and the canal complex surface was similar to that of a 36-hour (extended) treatment. The fibrils in Fig. 13b were taken to be cellulosic because cellulose is resistant to the treatments employed (Table VI). The porous region

Figure 11. Pit Fields in Surface Replicas of the Canal Complex

PF: Pit field — the whole circular area is the pit field and contains numerous small openings.

Plate Numbers: a. 6644AF
b. 6630AF

Magnification: 10,300X

Tissue Treatment: a. Untreated.
b. Soaked in ferric chloride for 6 hours and washed.

(M) shows an intercellular membrane where the amorphous and noncellulosic fibrillar structures have been removed.

In addition to the disappearance of identifiable pit fields (Fig. 11), elevated cross walls (Fig. 12) were not observed on the canal complex surface after extended acid or potassium ferrocyanide treatment. The origin or function of the elevated structures was not determined, but they were observed throughout the untreated canal complex surface and in some ultrathin sections (Fig. 22). Figure 14 illustrates an intermediate stage in the disappearance of these structures. In addition to the removal of soluble material, the disappearance of the elevated structures was also attributed to tissue swelling during the washing treatment. As tissue swelling stretched the membrane remnants over the cross wall interfaces, the elevated effect was eliminated. The pitlike depressions shown in Fig. 14 were common in tissue subjected to 6 and 12-hour acid treatments but not after extended treatment. These structures were associated with longitudinal wall interfaces and apparently represent weak or acid-sensitive points in the multilayered membranes.

The existence of noncellulosic fibrils in the surface of the canal complex was not proven but only indicated by Fig. 12-14. Although there is no reason to believe


a
PF
1um


b
PF
1um

Figure 12. Surface Replicas of an Untreated (a) and Hydrochloric Acid Treated (b) Canal Complex

A: Canal complex axis
E: Elevated cross wall
H: Hole in the replica
L: Location of a longitudinal wall interface

Plate Numbers: a. 5393AF
b. 6053AF

Magnification: 7200X

Tissue Treatment: a. Untreated.
b. Soaked in hydrochloric acid for 6 hours and washed.

that the disappearance of fibrillar material was an artifact attributable to specimen preparation, this is always a possibility with electron microscopy. Therefore, the uplifted fibrils were examined to determine whether their structure and dimensions were similar to cellulosic fibrils (46, 47).

Figure 15a shows fibrillar material that has a distinct ladderlike structure. Cross-bars, or rungs of the "ladder" appear to be evenly spaced and do not appear to be associated with an underlying structure. In Fig. 15b, some of the rungs are attributable to fibrils in the lower, intact layer indicating that structural aspects of the uplifted fibrils are only visible when the underlying structure is unshadowed or nonexistent. The ladderlike structures in no way correspond to the essentially solid, cellulose fibril built up of aggregated elementary fibrils (46, 47). Although the dimensions of the noncellulosic (ladderlike) fibrils appear to vary, this is due in part to fibril aggregation. Examination of Fig. 15b shows that separation of adjoining fibrils causes the apparent branching and much of the variation in fibril diameter. Cross-sectional shape (unknown) could also explain the variation in fibril diameter. The following dimensions were based on the


a
A
H
E
2μm

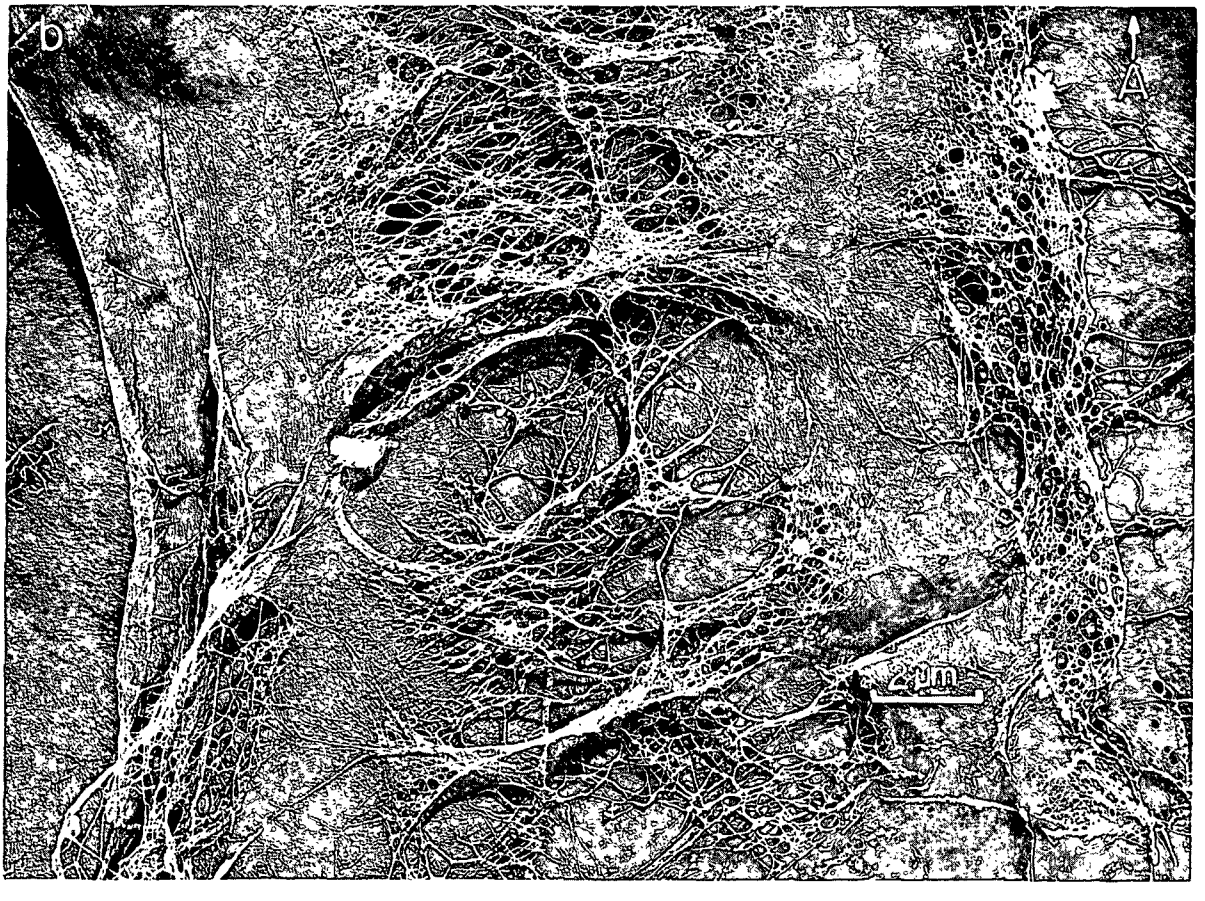

b
A
2μm

Figure 13. Surface Replicas of Treated Canal Complexes

A: Canal complex axis
L: Location of a longitudinal wall interface
M: Intercellular membrane

Plate Numbers: a. 5484AF
b. 6231AF

Magnification: a. 9400X
b. 12,900X

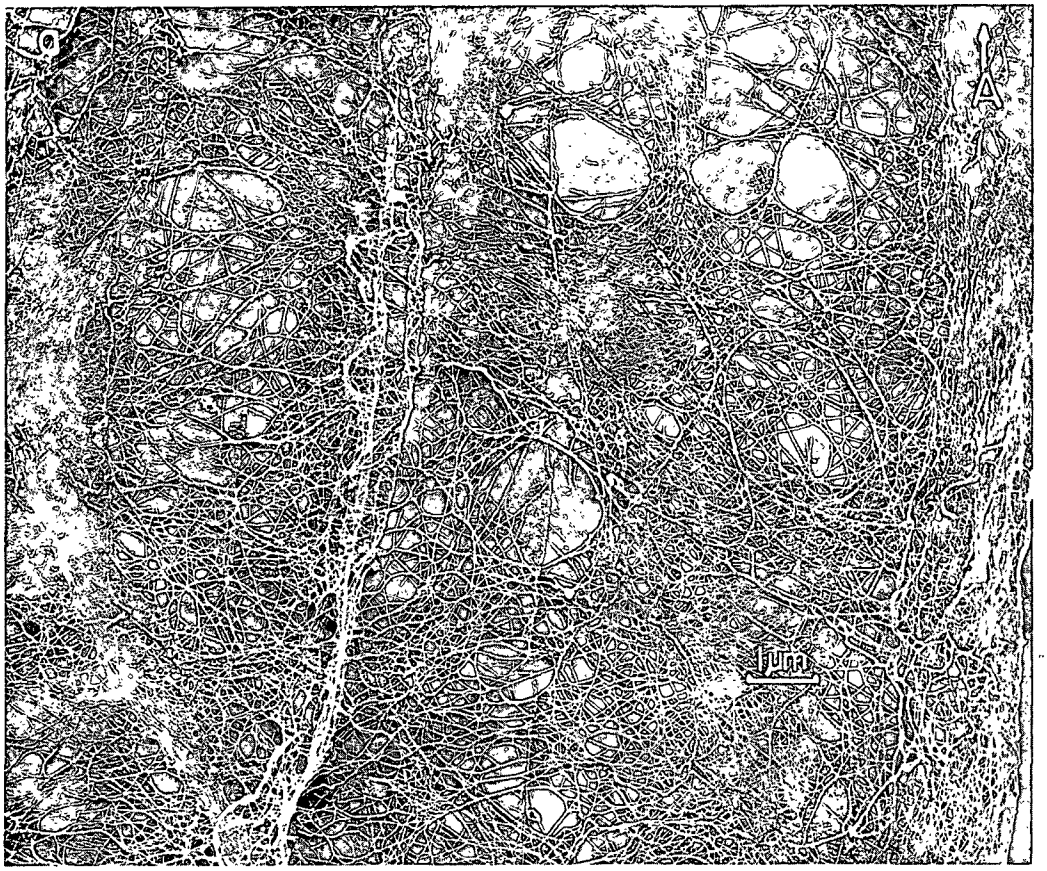
Tissue Treatment: a. Soaked in potassium ferrocyanide at pH 2.4 for 12 hours and washed.
b. Soaked in potassium ferrocyanide at pH 6.8 for 12 hours and washed. Similar in appearance to a surface replica of tissue treated for 36 hours.

larger fibrils, assuming an oval or rectangular cross section. Units are in nanometers (1×10^{-9} m.).

Fibril width	35
Thickness of "ladder" sides	9
Thickness of "ladder" rungs	9
Distance between rungs	25-30

These are approximate values which may vary with decomposition resulting from chemical treatment and may not represent true dimensions of the intact structures.

The noncellulosic fibrils were only distinguished from cellulosic structures in those parts of the surface replicas not completely shadowed with palladium. These areas were relatively common and extensive on the canal complex surface as considerable amounts of ray and radial canal tissue debris were present (Fig. 5). When the palladium source (13) was placed at an angle of 30 degrees to the plane of the canal complex surface, areas directly behind the surface contaminants were


a
A
1μm


b
A
M
A
1μm

Figure 14. Surface Replica of a Hydrochloric Acid Treated Canal Complex Showing the Effects of a Short-Term Treatment

A: Canal complex axis
E: Elevated cross wall
F: Uplifted fibrils
L: Location of longitudinal wall interfaces
PD: Pitlike depression

Plate Number: 5919AF

Magnification: 4300X

Tissue Treatment: Soaked in hydrochloric acid for 6 hours and washed.

not shadowed. Consequently, these areas were structureless when viewed with the electron microscope. However, because noncellulosic fibrils in these areas were often above the tissue surface, they were sometimes partially covered with palladium. An uplifted, noncellulosic fibrillar network, of continuous overshadowed and unshadowed areas is shown in Fig. 16. The uplifted strands in the unshadowed areas are unique and represent the noncellulosic fibrils. In contrast, the uplifted structures in the shadowed areas cannot be distinguished from cellulosic fibrils. These features are illustrated in Fig. 17, where selected areas of Fig. 16 are highly magnified. The uplifted strands in Fig. 17a appear structureless, as both the upper fibril surface and the tissue surface directly below are covered with palladium. Structural features in the two surfaces are therefore superimposed, and no details are visible. The fibrils appear light colored because most of the electrons were reflected by the double layer of palladium. The uplifted, noncellulosic fibrils are considerably thicker than those in the lower surface. This is particularly evident where the two layers merge and illustrates another difference between these structures and cellulosic fibrils. Figure 17b shows how structural detail within the uplifted fibrils increases as the degree of shadowing on the underlying surface decreases. This illustrates both the unique ladderlike noncellulosic fibrils and fibril decomposition.

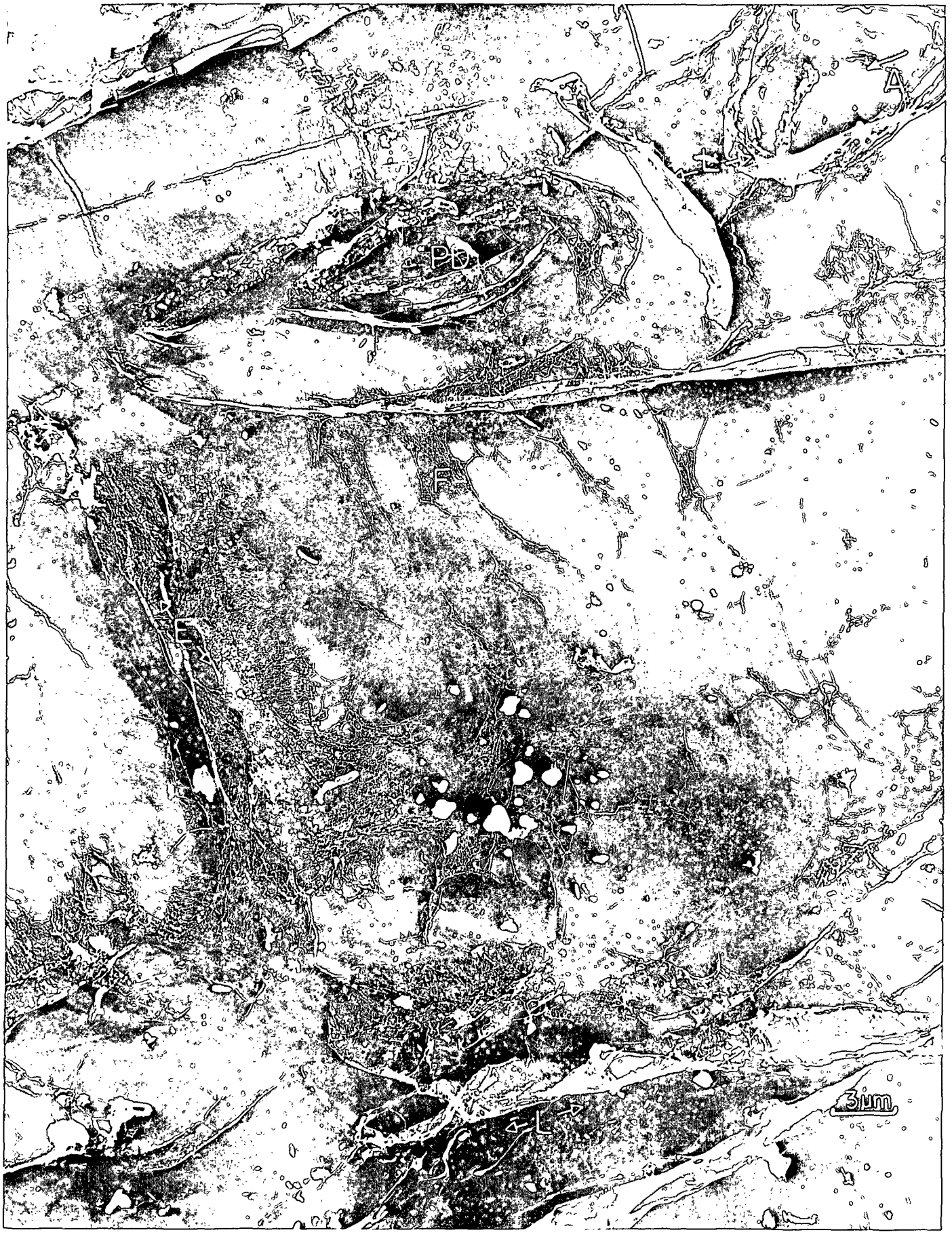

A
PD
F
E
3μm

Figure 15. Surface Replicas of Treated Canal Complexes Showing the Ladderlike Structure of the Noncellulosic Fibrils

F: Noncellulosic fibrils.

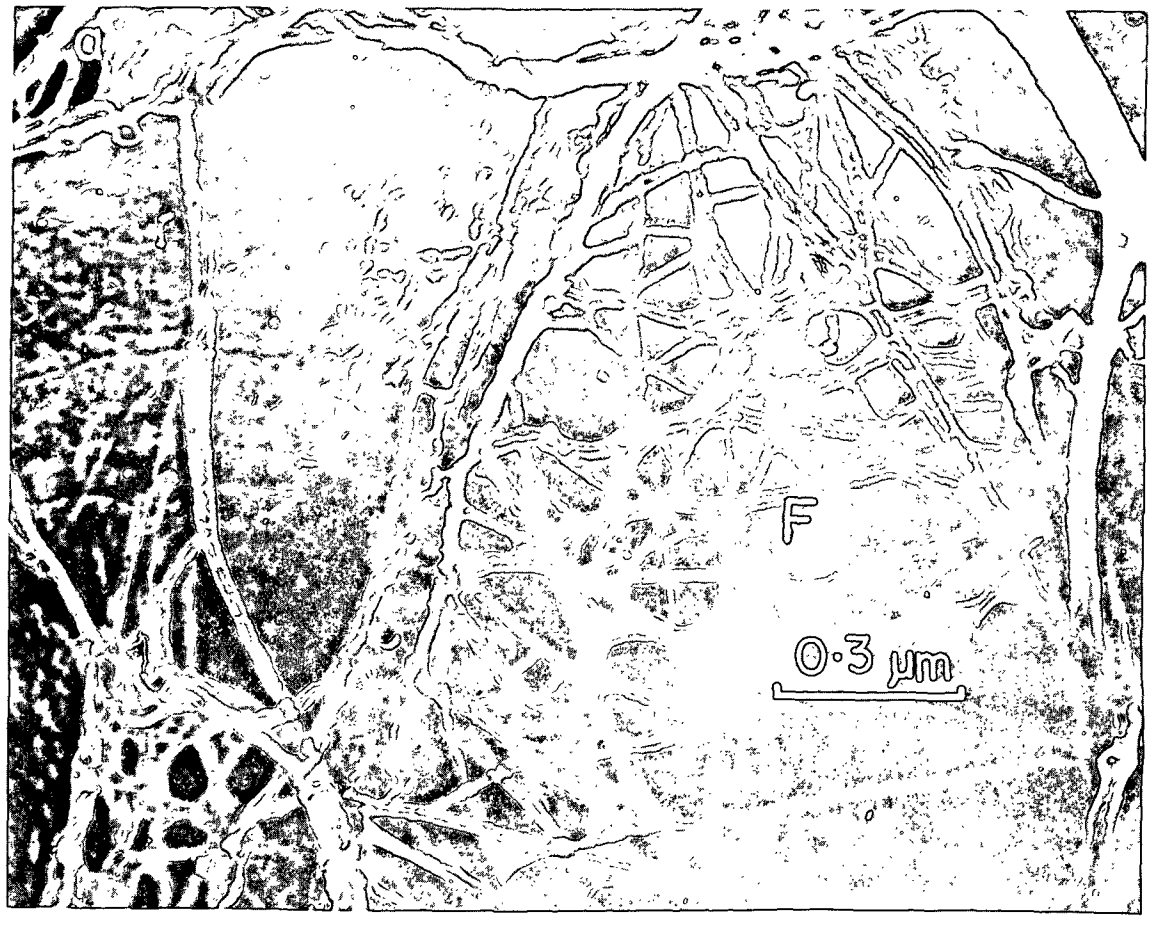
Plate Numbers: a. 6016AF
b. 6441AF

Magnification: a. 82,500X
b. 60,000X

Tissue Treatment: a. Soaked in potassium ferrocyanide at pH 2.4 for 12 hours and washed.
b. Soaked in hydrochloric acid for 12 hours and washed.

In an attempt to verify the existence of noncellulosic fibrils, purified cotton fibers (48) (98% glucan) were treated and observed by the procedures employed with the canal complexes. Fibrils with a chemical sensitivity and structure similar to those observed in surface replicas of the canal complex were not observed.

Roelofsen (1, 2), Mühlethaler (47), and Marchessault and Sarko (49) reviewed the distribution and structure of noncellulosic fibrils in plants. Although Roelofsen and Kreger (50) have published the only work which describes noncellulosic fibrils in the cell walls of higher plants, their work has never been accepted. This has been attributed to the possibility that their short, aggregated pectic fibrils may have resulted from the chemical treatment (2). Data obtained during the current study (Table VI) support the conclusions of Roelofsen and Kreger, as the disappearance of fibrillar material in the canal complex was associated with the loss of galacturonic acid and multivalent cations. If the polarization and x-ray techniques of Roelofsen and Kreger were applied to the canal complex fibrils, the conclusions of the two studies would probably be verified.


a
F
0·3 µm

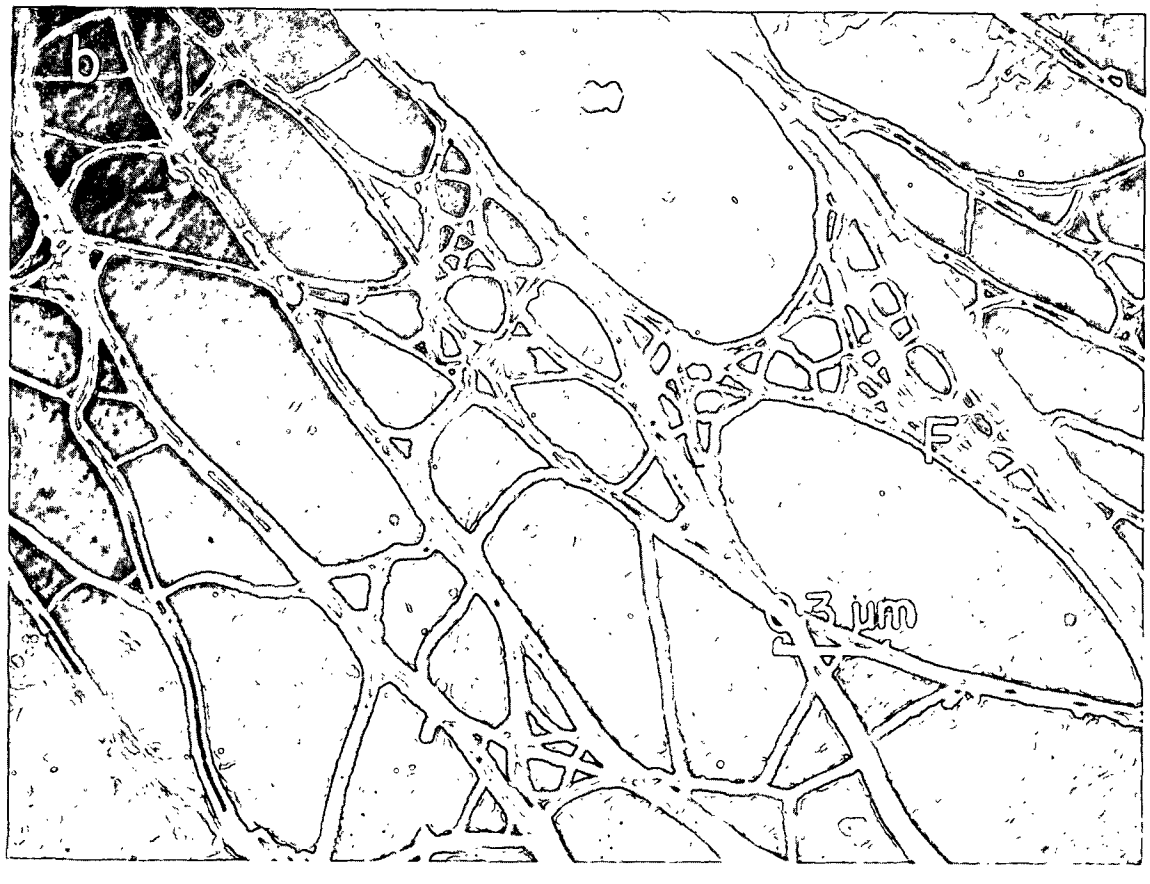

b
F
3 µm

Figure 16. Surface Replica of a Treated Canal Complex Showing Unshadowed and Palladium Shadowed Areas

N: Area not shadowed with palladium
S: Area shadowed with palladium

Plate Number: 6224AF

Magnification: 10,500X

Tissue Treatment: Soaked in potassium ferrocyanide at pH 6.8 for 12 hours and washed.

Any structure proposed for the canal complex fibrils must account for their apparent stability after practically all calcium in the tissue is replaced with the ferric cation (Fig. 11). As calcium is directly associated with the fibrils (the removal of calcium causes fibril disintegration), this would change the crystalline properties but not necessarily the fibril outline as viewed under the electron microscope. The amorphous matrix in which the fibrils are embedded is stabilized by calcium or ferric cations and would tend to resist morphological changes.

Cell Readhesion

Adjacent cell walls of the tissue treated with potassium ferrocyanide separated when soaked in water. When this material was subsequently soaked in a solution containing multivalent cations, the tissue was strengthened, and adjacent cell walls were readhered, at least partially (Fig. 18). This effect was obtained with solutions of ferric chloride (pH 1.9), calcium acetate (pH 4.5), and uranyl acetate (pH 4.5). The outline (diameter) and strength of a readhered, washed, hydrochloric acid-treated canal complex was identical to that of untreated tissue (Fig. 18a). Because canal complexes, which were treated in hydrochloric acid or potassium ferrocyanide and subsequently washed, have similar chemical compositions (Table VI), differences in the readhered tissues were attributed to the degree of cell separation


Z
Z
Z
1 µm

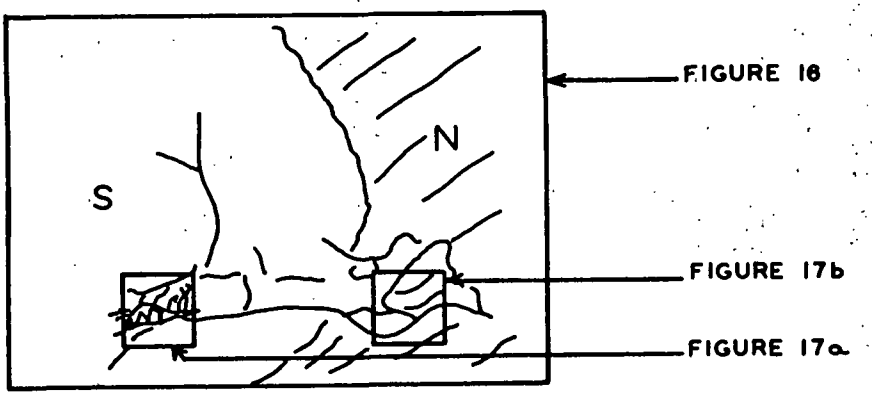
Figure 17. Surface Replicas of a Treated Canal Complex Showing Highly Magnified Areas of Fig. 16. (See Diagram Below)

D: Decomposing noncellulosic fibril
L: Ladderlike noncellulosic fibril
N: Area not shadowed with palladium
S: Area shadowed with palladium

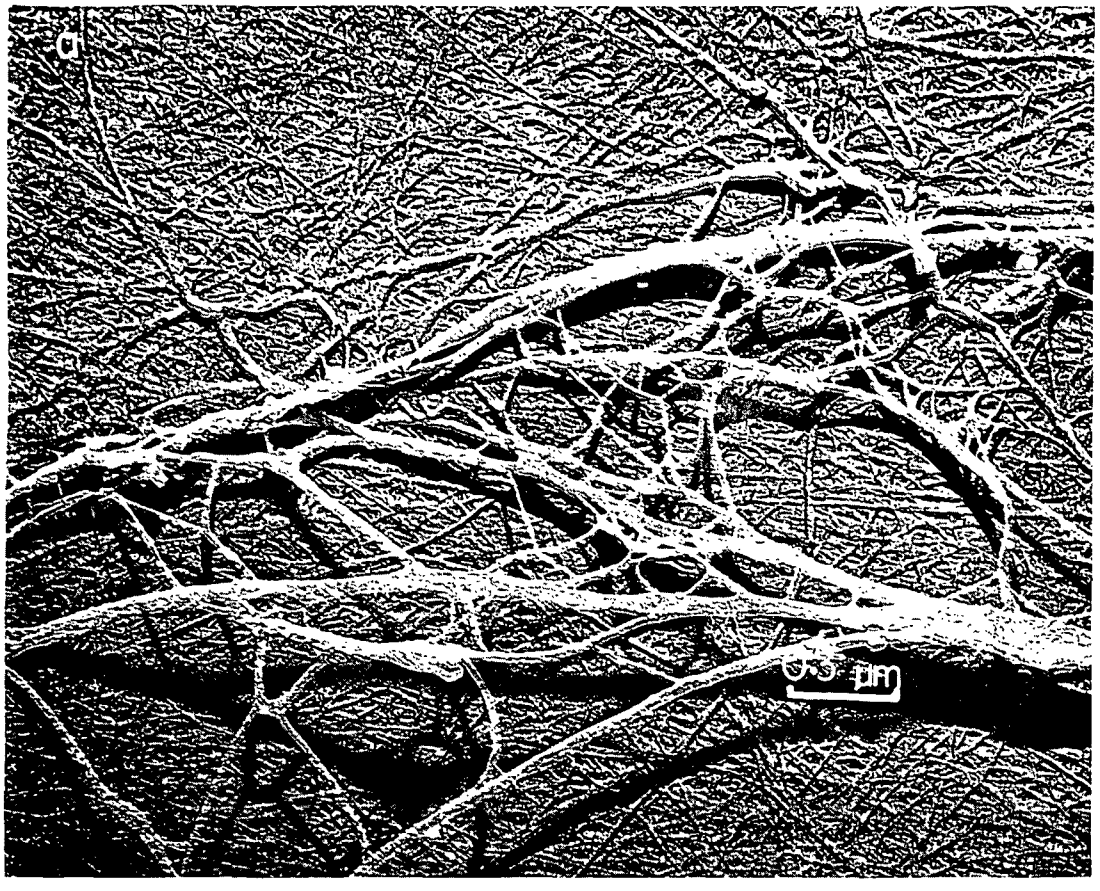
Plate Numbers: a. 6225AF
b. 6226AF

Magnification: 49,000X

Tissue Treatment: Soaked in potassium ferrocyanide at pH 6.8 for 12 hours and washed.



prior to treatment with multivalent cations. Ginzberg (6) studied the ability of multivalent cations to recement cells of Alaska pea seedling root tips treated with ethylenediaminetetraacetic acid. He concluded that the removal of multivalent cations was reversible. However, Taylor and Wain (9) and the data in Table VI show that large quantities of pectic substances are extracted with the cations, and, therefore, cell separation or the removal of cations is not completely reversible. The electron microscope work also supports this conclusion.


a
0·3 µm

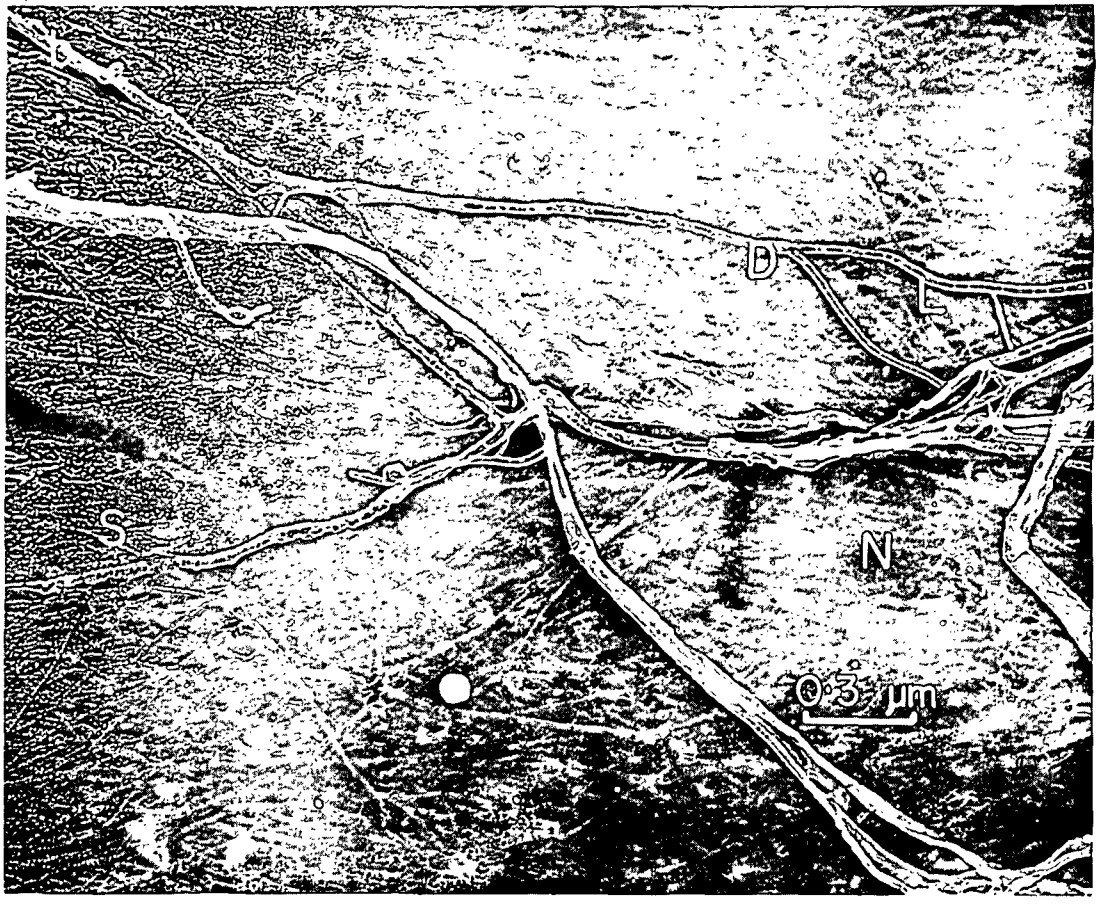

D
L
S
N
0·3 µm

Figure 18. The Ability of a Ferric Chloride Solution to Restrengthen Canal Complexes Treated with Potassium Ferrocyanide

a. An untreated canal complex with a cover glass applied.
b. A canal complex which was treated with potassium ferrocyanide and subsequently washed.
c. The same canal complex as in (b) after a cover glass was applied.
d. Canal complexes which were treated with potassium ferrocyanide, washed, and subsequently soaked in a ferric chloride solution. Cover glasses were applied after soaking the complexes in water.

Plate Numbers: a. 68M-636R
b. 68M-636AD
c. 68M-636AE
d. 68M-636AK

Magnification: 30X

Tissue Treatment: a. Untreated.
b. Soaked in potassium ferrocyanide at pH 2.4 for 12 hours and washed.
c. Soaked in potassium ferrocyanide at pH 2.4 for 12 hours and washed.
d. Soaked in potassium ferrocyanide at pH 2.4 for 12 hours, washed, and subsequently soaked in ferric chloride at pH 1.9 for 6 hours and washed.

See also Fig. 5.

Cell readhesion was studied at different ultrastructural levels. Intercell-wall, interlamella, and interfibril adhesion were observed when tissue treated with potassium ferrocyanide was washed and soaked in a ferric chloride solution. The washed, hydrochloric-acid treated tissue, after treatment with ferric chloride, was similar but not identical to the untreated complex. The maximum extent of wall, lamella, and fibril separation observed in canal complexes which were treated with potassium ferrocyanide, washed, and then soaked in a ferric chloride solution, is shown in Fig. 19. Figure 23a shows a more typical electron micrograph where the wall lamellae are closely packed. Figure 19 also represents the minimum degree of

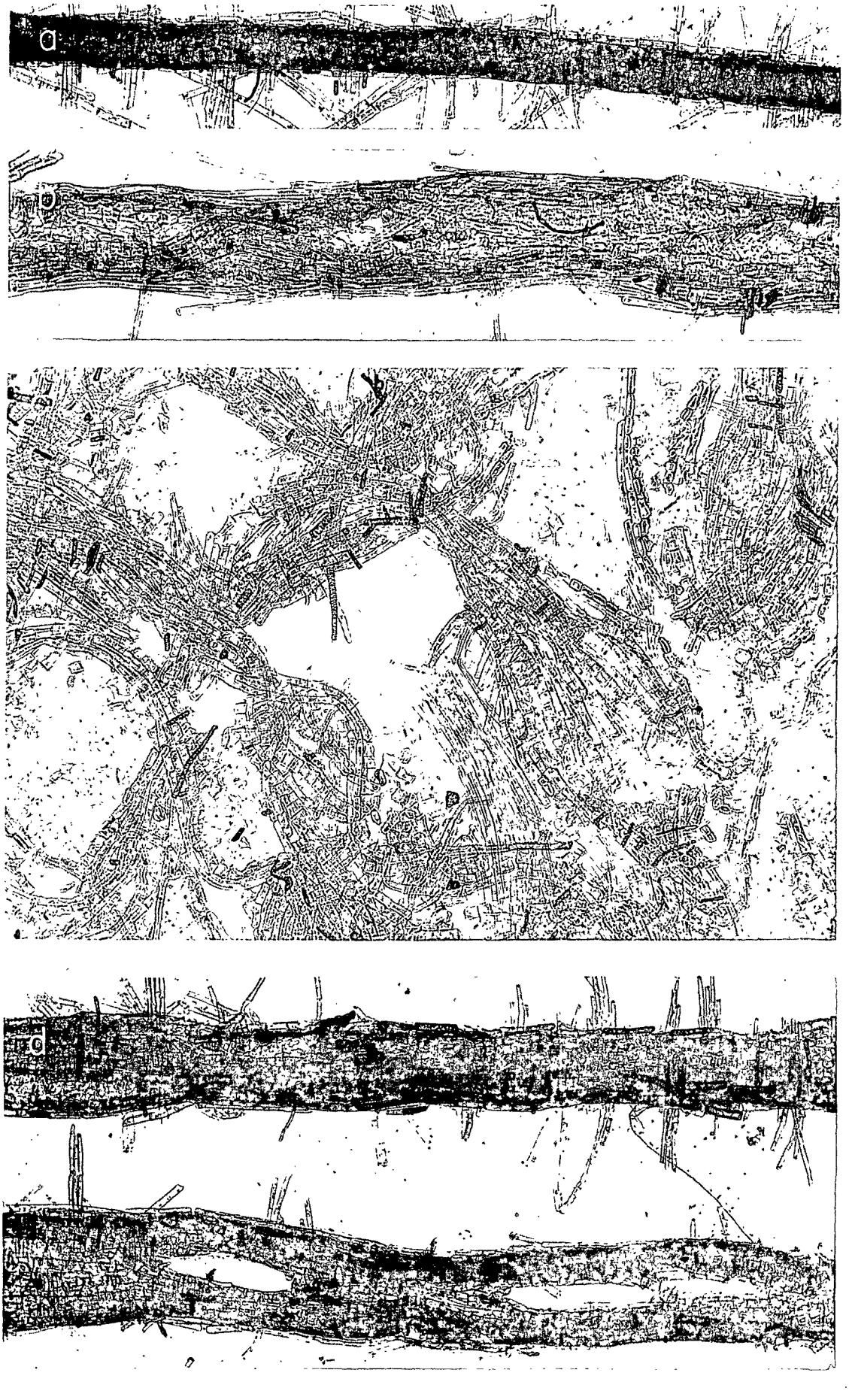

a
b
c

Figure 19. Shadowed, Ultrathin Cross Section of a Canal Complex Treated with Potassium Ferrocyanide and Restrengthened by Soaking in a Ferric Chloride Solution

F: Intercell-wall fibrils
I: Intermediate cell
M: Intercellular membrane
X: Separation due to shrinkage (see text)

Plate Number: 6802AF

Magnification: 5500X

Tissue Treatment: Soaked in potassium ferrocyanide at pH 6.8 for 36 hours, washed, soaked in ferric chloride at pH 1.9 for 6 hours, and washed again.

See also Fig. 10b and 23a.

separation observed in a similarly treated canal complex without the ferric chloride post-treatment. Maximum separation observed in such tissue is illustrated in Fig. 10b. The appearance of separated cell walls, lamellae, and fibrils in Fig. 19 indicates that readhesion occurs only where adjacent wall elements are in close proximity when subjected to treatment with ferric chloride. The effect of shrinkage of the embedding medium on cell wall ultrastructure is also illustrated in Fig. 19. Areas marked "X" show the parts of the wall separated by shrinkage. No intercell-wall fibrils are visible in these regions as they were readhered by the cation treatment. Separation occurred between the more weakly bonded walls or wall lamellae. Where intercell-wall fibrils (F) are visible, adjacent wall elements were not bonded by the cation treatment and were pulled further apart as the embedding medium polymerized. This is illustrated in Fig. 10b where the cation treatment was omitted.

The effects of the ferric chloride post-treatment on cell wall ultrastructure were more apparent in shadowed longitudinal sections than in shadowed cross sections. When washed, potassium ferrocyanide treated tissue was soaked in a ferric chloride


M
X
F
3 µm

solution, cell wall identity was retained (Fig. 20b). However, if the cation treatment was omitted, the cell wall appeared as an unorganized fibrillar mass (Fig. 20a). After the embedding medium was removed from the longitudinal sections, the short fibril remnants which were arranged in distinct lamellae, fell on their sides (Fig. 21). Fibrils within the lamellae were directed approximately perpendicular to the plane of the electron micrograph before the embedding medium was removed. The scattered and disorganized arrangement of the fibril remnants in Fig. 21b was evidently due to polymerization and the subsequent removal of the embedding medium. Shrinkage from polymerization separated adjoining wall elements, and movement of the section in and out of the butyl methacrylate solvent (chloroform) probably caused most of the lamellae disintegration. These effects were not observed in sections of similarly treated tissue soaked in a ferric chloride solution because

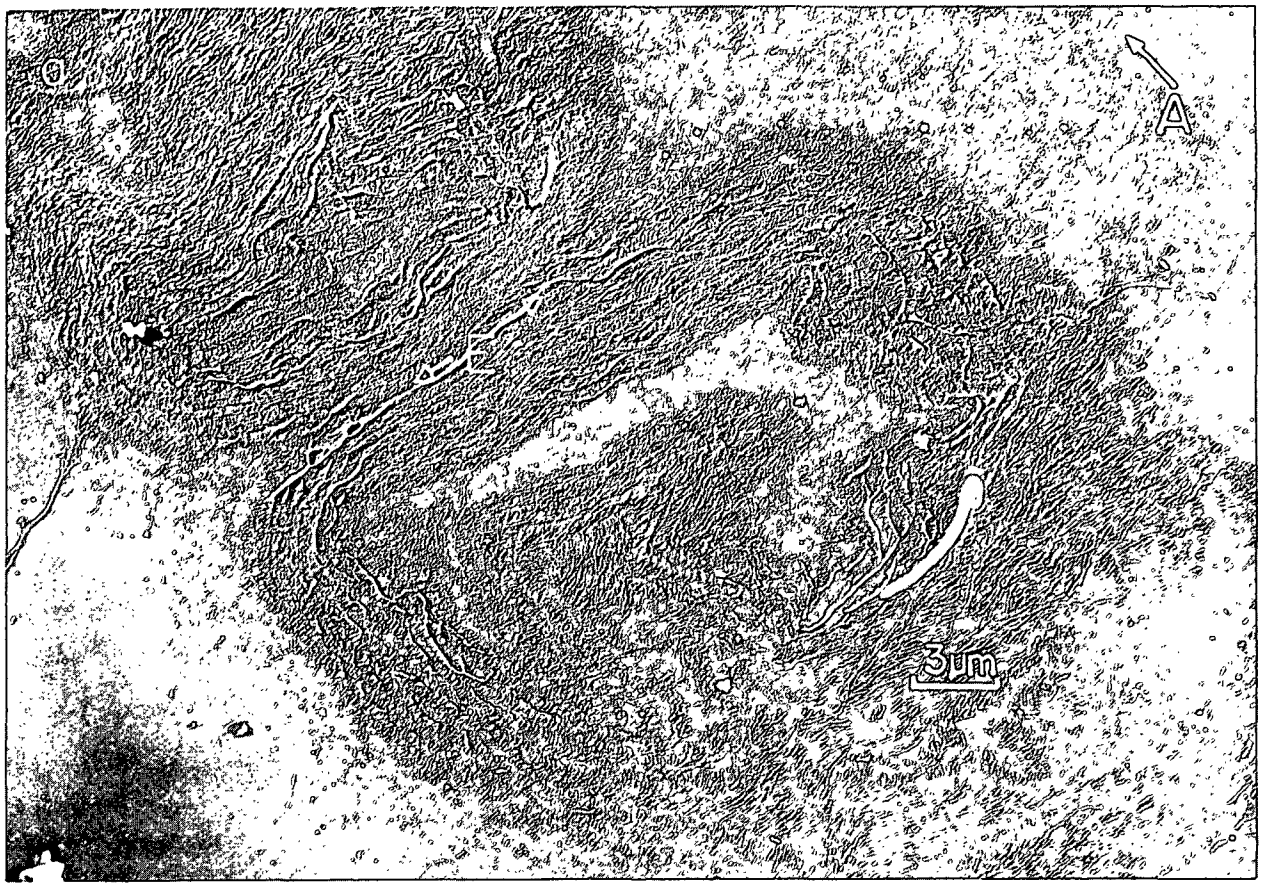
Figure 20. Shadowed, Ultrathin Longitudinal Sections of a Canal Complex Treated with Potassium Ferrocyanide. Figure 20b Illustrates that Adjacent Wall Elements are Bonded and that Cell Wall Identity is Retained when the Tissue Residue is Washed and Treated with Ferric Chloride

A: Canal complex axis
E: Cross walls
L: Separated longitudinal walls

Plate Numbers: a. 6788AF
b. 6861AF

Magnification: a. 3600X
b. 2800X

Tissue Treatment: a. Soaked in potassium ferrocyanide at pH 6.8 for 36 hours and washed.
b. Soaked in potassium ferrocyanide at pH 6.8 for 36 hours, washed, soaked in ferric chloride at pH 1.9 for 6 hours, and washed again.


a
A
3μm

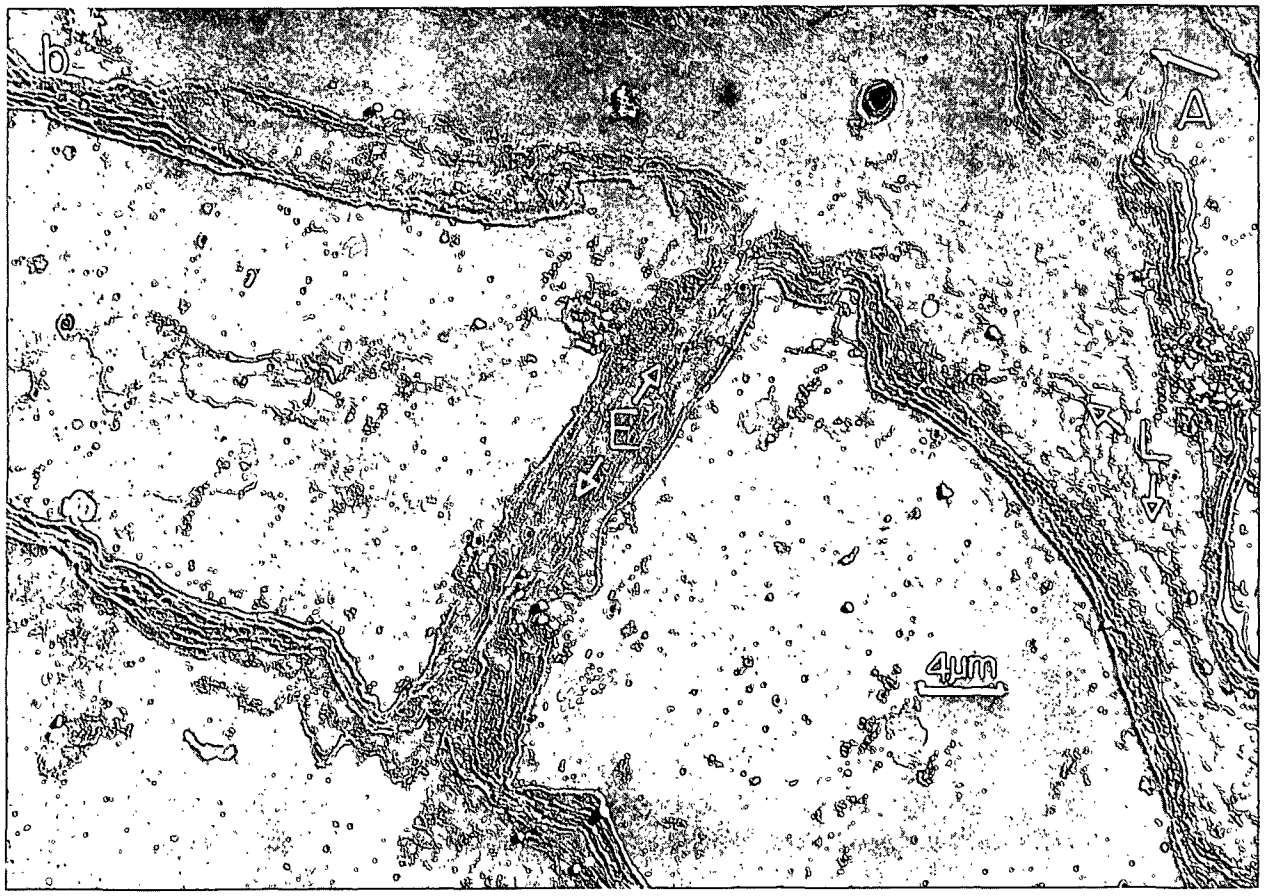

b
A
4μm

Figure 21. Shadowed, Ultrathin Longitudinal Sections of a Canal Complex Treated with Potassium Ferrocyanide

A: Canal complex axis
L: Wall lamellae which have fallen on their sides after removal of butyl methacrylate.

Plate Numbers: a. 6842AF
b. 6854AF

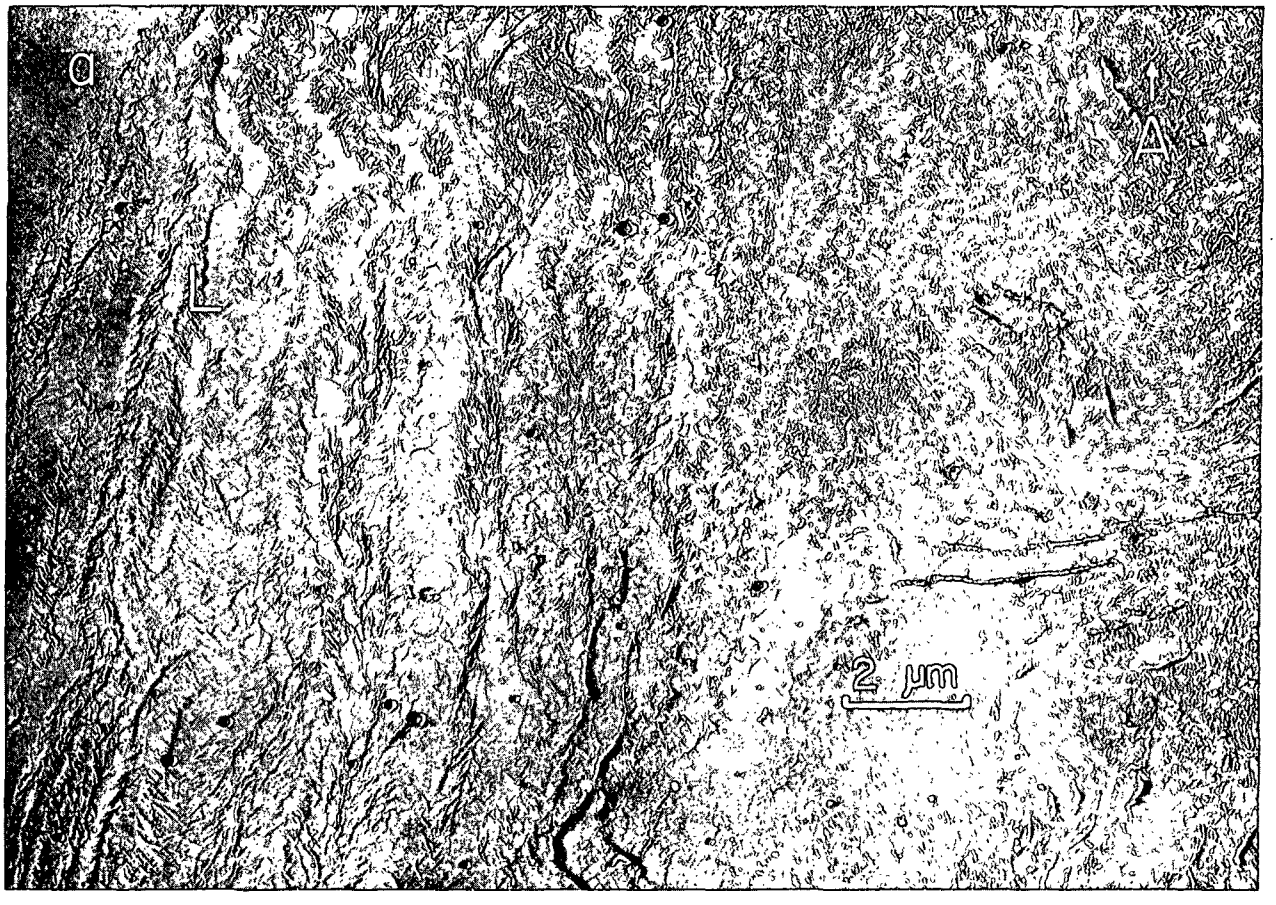
Magnification: a. 8400X
b. 16,800X

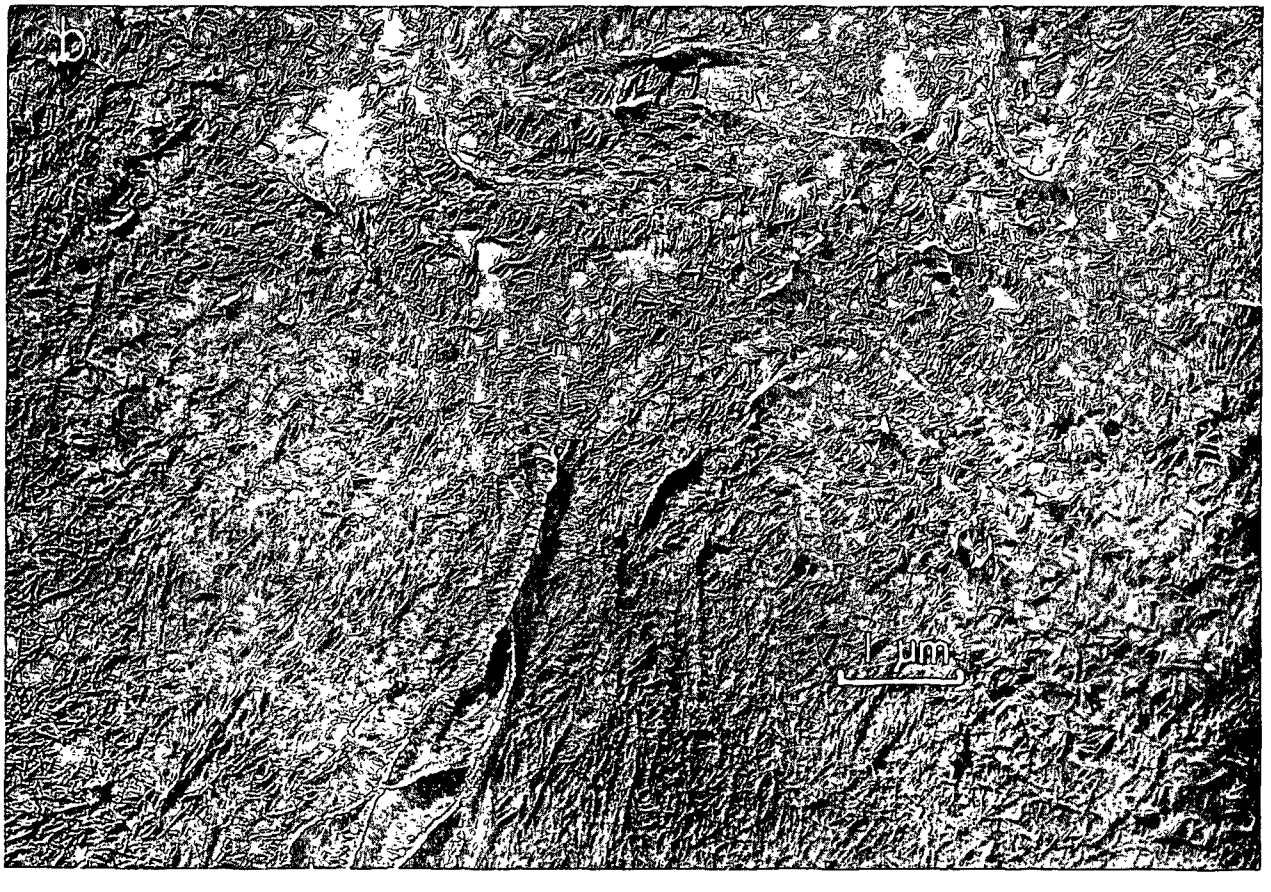
Tissue Treatment: Soaked in potassium ferrocyanide at pH 6.8 for 36 hours and washed.

wall elements in close proximity were bonded and, therefore, not separable by effects associated with the embedding medium. Lamellae disintegration (Fig. 21b) was also absent in the shadowed, cross sections as most of the fibrils were directed in the plane of the section and, hence, were not cut into short lengths during sectioning.

Stained, ultrathin sections which were not complicated by shrinkage or removal of the maraglas embedding medium, confirmed conclusions based on observations of the shadowed, ultrathin sections. The degree of separation between adjacent cell walls and within the cell walls of canal complexes treated with potassium ferrocyanide and then washed (Fig. 9) was unchanged when this tissue was subsequently soaked in a ferric chloride or uranyl acetate solution.

A number of factors associated with particle flocculation in colloidal dispersions may be associated with readhesion of separated canal complex cells. For this reason, the following description of phenomena associated with flocculation is included (45). Particles in a colloidal dispersion only flocculate when the attractive forces between them exceed the repulsive forces. Although the repulsive potential is markedly affected by the surrounding medium, the attractive potential is


a
A
L
2 μm


b
1 μm

relatively independent of this medium and depends primarily on particle composition. Repulsive forces are associated with an electrical charge on the surface of the particle which, when in contact with an aqueous electrolyte, induces a redistribution of the ions in solution. This results in the formation of an electrical double layer which can be collapsed and the surface potential destroyed by increasing the electrolyte concentration. Under these circumstances, when two lyophobic particles come into close proximity, they flocculate because the attractive forces exceed the repulsive forces. The situation is complicated with lyophilic particles because the dispersion medium often adheres strongly to particle surfaces and is not easily displaced. The surfaces of fibrils in the walls of the canal complex cells, like cellulose (45), probably exhibit some properties of both lyophilic and lyophobic particles. However, in contrast to particles in a colloidal dispersion, wall elements are arranged and held in a three-dimensional fibrillar network and are not in a constant state of agitation or Brownian motion.

Cell separation occurred when canal complexes treated with potassium ferrocyanide were soaked in water. Because cell separation has been attributed to the expansion of an electrical double layer (page 30), it is possible that flocculation of adjacent wall elements and cell walls is associated with cell readhesion. Examination of the restrengthened tissue with the electron microscope showed that adjacent wall elements were only bonded together where they were in close proximity when treated. This observation is in accordance with phenomena described previously in this thesis. When the treated tissue (potassium ferrocyanide) was soaked in water, potassium ions associated with the surface charge were replaced by the hydronium ion. This occurs only when the potassium concentration in the washing medium is very low and when the electrical double layer has reached maximum thickness (Fig. 6). When such tissue is subsequently soaked in a ferric chloride solution, the electrical double layer is collapsed (45). Because adjacent wall elements were not pulled

together as a result of this treatment, it is concluded that flocculating phenomena have an insignificant role in the readhesion of separated canal complex cells. The rigidity of the fibrillar networks in the expanded cell walls (Fig. 10b) prevents adjacent wall elements from coming into close proximity after the repulsive potential is removed. Consequently, distances between adjacent wall elements are greater than the range of their attractive potentials and flocculation does not occur. Lyophilic substances on the separated fibril surfaces are probably solvated and may be an additional factor in inhibiting flocculation of adjacent wall elements.

On the basis of the chemical composition data in Table VI, light and electron microscope examination, and the apparent absence of flocculation effects, it is concluded that the restrengthening effect of multivalent cations is probably associated with chemical bonding. The most prevalent bonds formed are probably those where the residual acidic substances on the surface of adjacent wall elements bond with the added cations. However, other types of bonding may occur, particularly the formation of complexes between cations and residual amorphous substances. Interfibril, interlamella, and intercell-wall adhesion occur, but only where adjacent wall elements are in close proximity prior to treatment with multivalent cations.

Technological Applications

Technological aspects of the readhesion study were considered with both the canal complex and pulp fibers (holocelluloses A and B — Table III). After the cells of canal complexes treated with potassium ferrocyanide or hydrochloric acid and subsequently washed, were manually separated, they were bonded together by the procedure described in the experimental section. Canal complexes were not bonded when pretreated with ferric chloride. This was consistent with the microscopic studies as intrawall bonding probably occurred within and over the surface of the tissue during the pretreatment. When the procedure was applied to washed,

hydrochloric acid-treated pulp fibers and intact canal complexes, they were also bonded together. The ability to bond pulp fibers with multivalent cations is significant as it could lead to new papermaking processes. Thompson and Andrews (51), who continued this work, have been able to increase considerably the wet strength of handsheets and a paper roll which was treated on an experimental paper machine.

CELL WALL ULTRASTRUCTURE

PRIMARY CELL WALL AND MIDDLE LAMELLA

Although the primary cell wall has been described and characterized on numerous occasions (1-5, 46, 52), a definition suitable for all situations and plant tissues is not available. Wardrop (4) discussed difficulties inherent in defining this structure and proposed that "the primary wall is that structure which encloses or enclosed the protoplast during the phase of growth." This is a broad definition which needs to be modified according to the emphasis of a particular study. For this thesis, Wardrop's definition was modified to read: The primary cell wall is that structure which encloses a protoplast in the mature canal complex.

Current concepts of the middle lamella or intercellular region are vague and poorly defined, because the region has not been adequately characterized with respect to boundaries, structure, and chemical composition. The middle lamella is currently described by others as an amorphous, isotropic, adhesive substance between adjoining cells. This layer in unlignified cells is composed mainly of pectic substances and associated cations (1-5, 46). The conclusion that the middle lamella in a newly formed cell plate (5, 52) is completely isotropic is questionable. Albersheim (5) describes the cell plate as being positively birefringent as soon as the cytoplasmic vesicles coalesce, and concludes that cellulose fibrils are deposited

in the cell plate as it grows toward the walls of the mother cell. However, this conclusion is based on the observations of Becker (53), which Roelofsen (1) interprets as indicating that cellulose is deposited only on the cell plate surfaces. Frey-Wyssling and Mühlethaler (52) concur with Roelofsen's interpretation, apparently from their own observations. An acceptable interpretation has yet to be found, however, as the above conclusions are based on birefringence data. Albersheim (5) states that if randomly directed fibrils exist in the cell plate, they would not display birefringence under plane-polarized light. Thus, new techniques or approaches are required to verify or eliminate the hypothesis that the middle lamella, typified by a newly formed cell plate, is isotropic.

Before structural or chemical aspects of the middle lamella can be determined, the boundaries must be defined. Kerr and Bailey (54) in 1934 defined the middle lamella or intercellular substance as a truly isotropic layer between adjacent primary cell walls. Apart from the doubtful nature of the term isotropic, this definition is probably only valid with reference to cambial and other meristem initials and to the partition wall between daughter cells. Recent work (13, 31, 55) indicates that the above definition is not even applicable to cambial daughter cells, since structures which are probably remnants of the cambial wall are observed on, or associated with, the radial surfaces of immature and mature tracheids. The existence of these structures, as well as the previously described conflicts associated with the isotropic nature of the cell plate indicate that the middle lamella should be defined in more general terms. Consequently, the middle lamella is redefined as the intercellular region between adjoining primary cell walls. This is a workable definition which is not hampered by the composition or thickness of the region. The term middle lamella is retained, as the modified definition concurs with current usage and staining reactions (1-5, 52) and because a new terminology would only cause further confusion. Mahmood (55), who only recently published his work,

proposed a definition in terms of the primary wall, ancestral walls, and the intercellular material. However, because of the current uncertainty associated with the middle lamella, a general definition based only on boundaries seems more logical. In this way, the composition, structure, and thickness of the middle lamella, like the primary wall, can be described according to the emphasis of a particular investigation.

The middle lamella and primary wall boundaries are visible in Fig. 22. In addition, the middle lamella is indicated in three different positions.

1. Between adjacent longitudinal primary walls (ML).
2. Over the canal complex surface (CS).
3. Between adjacent cross walls (E).

Figure 22. Stained, Ultrathin Longitudinal Section of an Untreated Canal Complex

A: Canal complex axis
C: Cytoplasmic remnant
CS: Canal complex surface and a sectional view of an elevated cross wall. The three dark lines adjacent to "CS" probably represent lamellae of ancestral walls.
E: Perforated cross walls
I: Intermediate cell
ML: Middle lamella
O: Outer cell
P: Primary cell wall

Plate Number: 6133F

Magnification: 5200X

See also Fig. 2.


W
E
S

The middle lamella between adjacent longitudinal primary walls and over the canal complex surface is considerably thicker than the wall itself (P). The canal complex surface is actually the canal complex-tracheid interface, and, therefore, the surface layer which covers the primary wall is actually part of the middle lamella. In these two regions (1 and 2 on p. 76), the limitations of stained, ultrathin sections are apparent. Although the thickness of the middle lamella is apparently due to the presence of ancestral walls, the lamellalike structures (CS), which are clearly visible, are not sufficiently resolved to confirm this hypothesis. Although the middle lamella between adjacent end walls (E) is not clear in Fig. 22, it is a thin region and typical of a "mature cell plate." Figure 23 confirms the existence of ancestral walls and indicates that adjacent walls and wall lamellae are connected by fibrillar networks. Cell doublets near the center of each micrograph are encapsulated by thick, multilayered ancestral walls. These doublets were

Figure 23. Shadowed, Ultrathin Cross Sections of Potassium Ferrocyanide (a) and Hydrochloric Acid (b) Treated Canal Complexes Clearly Illustrate the Existence of Intact Ancestral Wall Skeletons

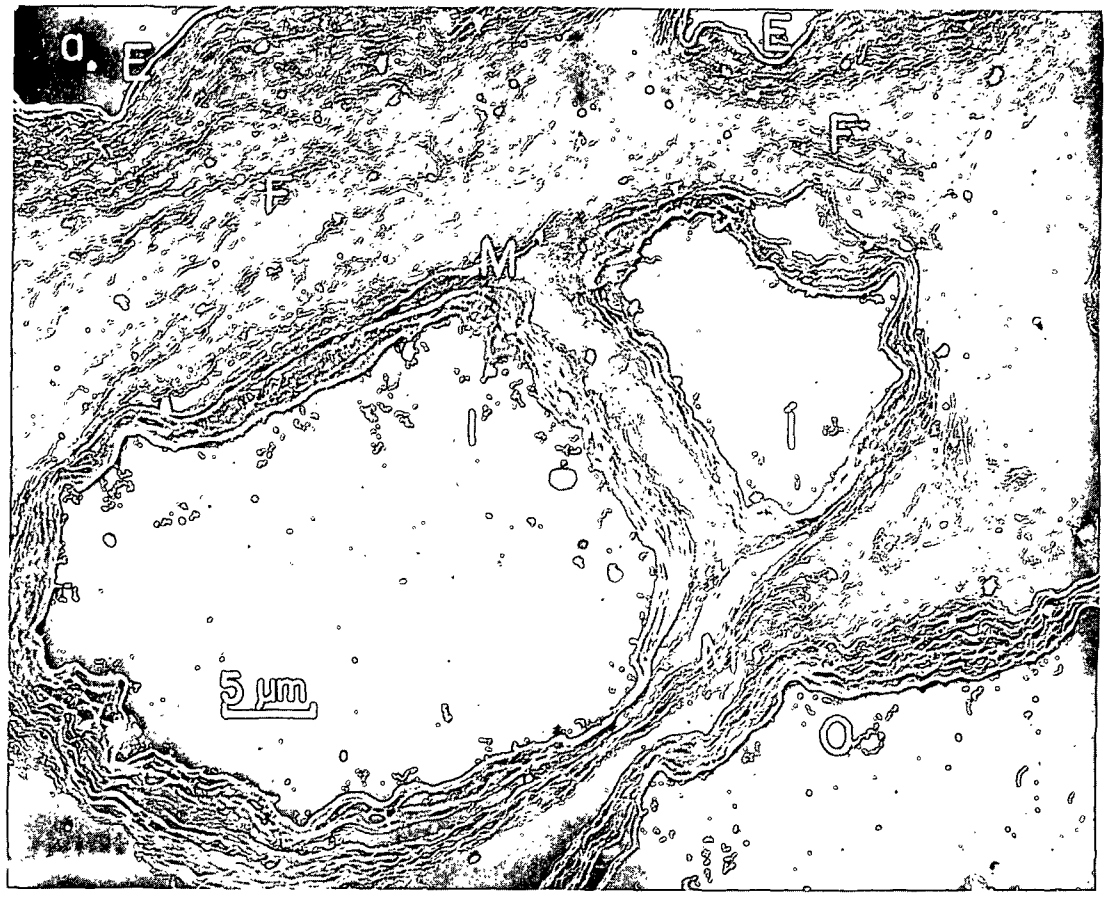
C: Cytoplasmic remnant
CS: Canal complex surface
E: Epithelial cell
F: Intercell-wall fibrils
I: Intermediate cell
M: Intercellular membrane
O: Outer cell

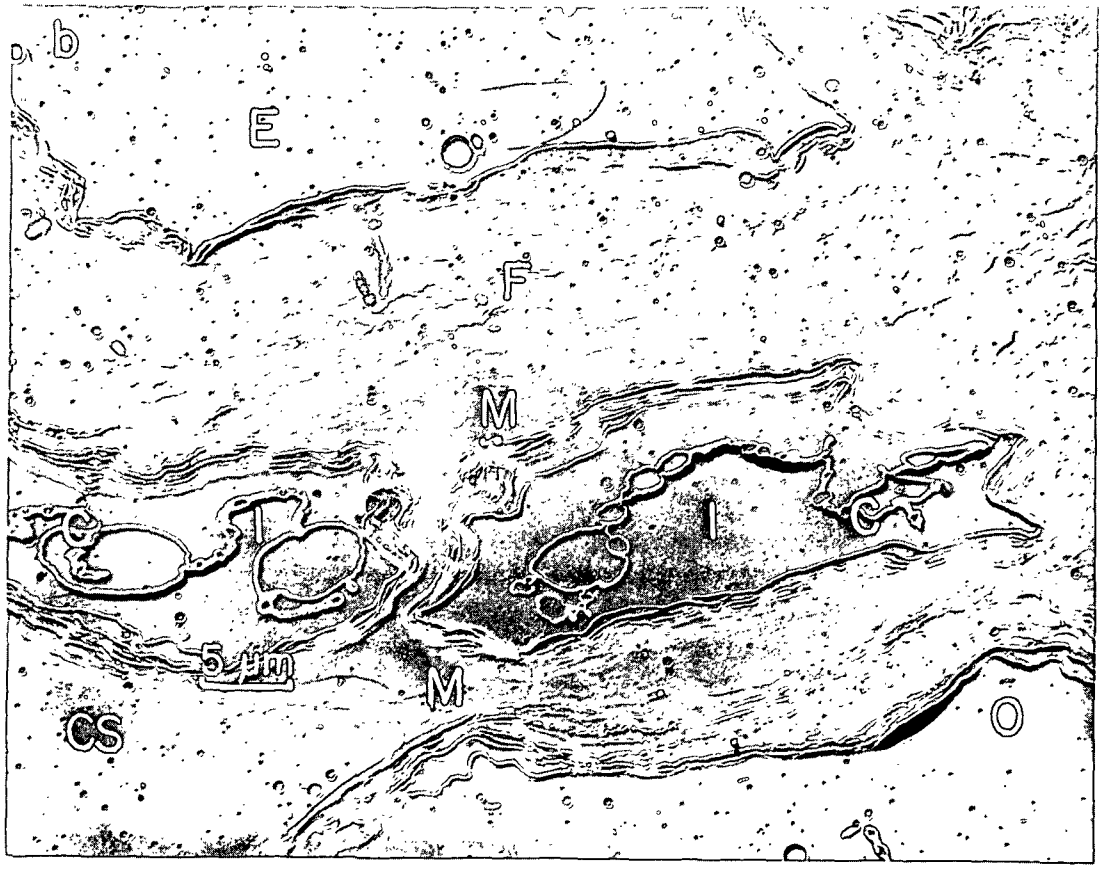
Plate Numbers: a. 6798AF
b. 6969AF

Magnification: 2500X

Tissue Treatment: a. Soaked in potassium ferrocyanide at pH 6.8 for 36 hours, washed, soaked in ferric chloride for 6 hours, and washed again.
b. Soaked in hydrochloric acid for 6 hours and washed.

See also Fig. 10, 19, and 22.


a.
E
E
F
F
F
M
I
I
5 µm
O


b
E
F
M
I
5 µm
M
CS
O

very common but were only found associated with intermediate cells. Ancestral walls (intercellular membranes) were observed surrounding all three cell types, but those associated with outer and epithelial cells were thinner and more readily ruptured. Figure 23 illustrates that, although the middle lamella and primary wall can be distinguished by staining [(5) and Fig. 22], they are structurally connected. Ancestral walls are often only distinguished from the primary wall where they cross intercellular interfaces. In addition, adjacent walls and wall lamellae appear to be connected by fibrillar networks. These features are more evident in Fig. 23b where the tissue was not subjected to a ferric chloride post-treatment.

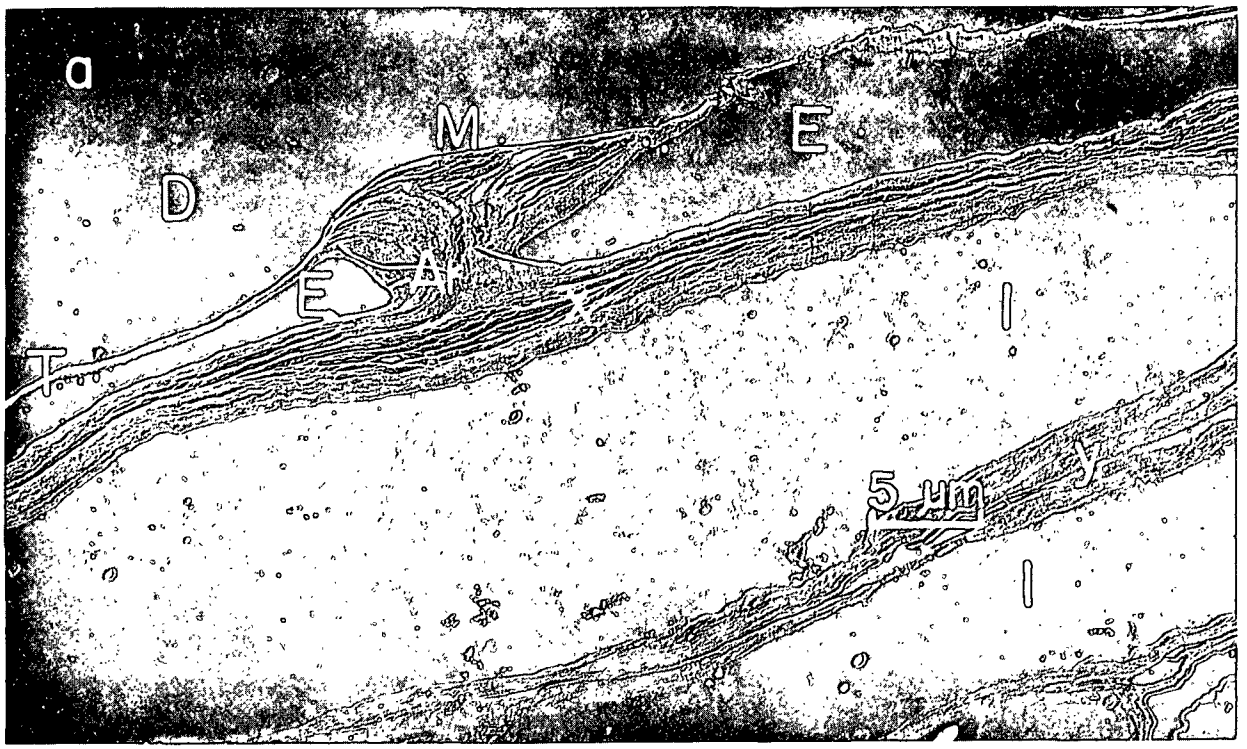
Shrinkage of the butyl methacrylate embedding medium during polymerization partially separated adjacent cell walls in untreated canal complexes. Figure 24 shows that cleavage occurs to a similar extent in the middle lamella (x) and along the middle lamella-primary wall interface (y). When these features are considered in conjunction with the existence of ancestral walls and fibrils which connect

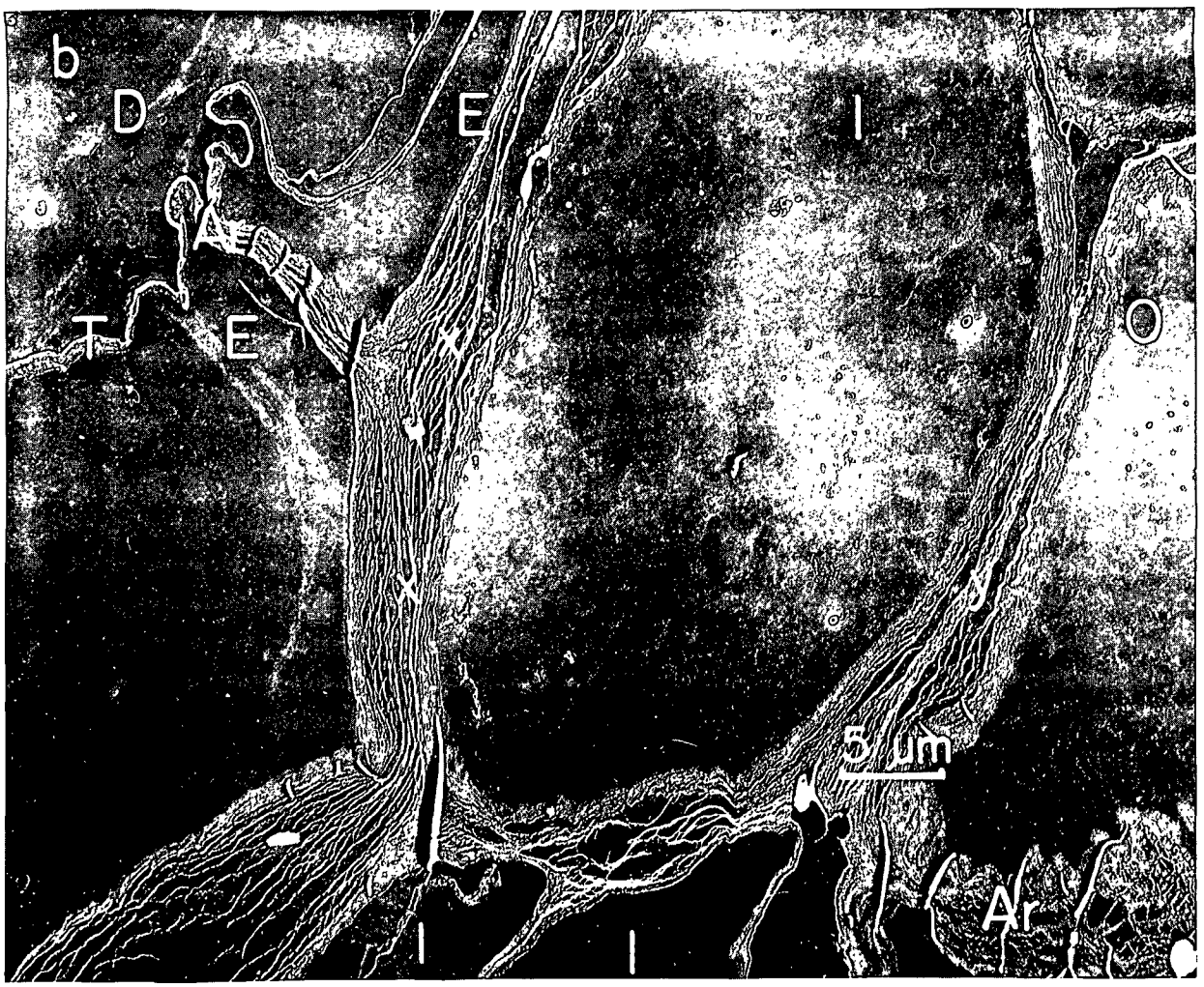
Figure 24. Shadowed, Ultrathin Cross Section of an Untreated Canal Complex Showing that Preferential Cell Separation Within the Middle Lamella Does not Occur

Ar: Artifact (Appendix X)
D: Canal
E: Epithelial cell
I: Intermediate cell
M: Intercellular membrane
O: Outer cell
T: Thin-walled portion of an epithelial cell
x: Cleavage within middle lamella
y: Cleavage along middle lamella-primary wall boundary

Plate Numbers: a. 6718AF
b. 6723AF

Magnification: 3000X


a
M
E
D
E
A
I
T
5 µm
I


b
D
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E
O
5 µm
Ar
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adjacent wall elements (lamellae, ancestral walls, and cell walls), they eliminate the concept that the intercellular region between adjacent cell walls differs from the other interfaces. Further evidence to support this conclusion is obtained from the homogeneous appearance of the middle lamella when stained (5). This is independent of the number of ancestral walls within the middle lamella (Fig. 22). When most of the amorphous substances are removed from the canal tissue, adjacent walls and lamellae are separated to a similar extent (Fig. 21a). Separation of ancestral wall layers within the middle lamella occurred during the canal complex isolation process and was evident in surface replicas (Fig. 29, 30).

Albersheim and Killias (20), by treating plant tissue with alkaline hydroxylamine, showed the distribution of methylated uronides in the primary cell wall and the middle lamella. They showed that pectic substances are present in both areas but are concentrated in the middle lamella. These observations concur with the current study (page 48) which shows that while the boundaries of the middle lamella are clearly apparent (Fig. 8 and 22), some of the pectic substances are continuous over the primary wall-middle lamella interface. These data indicate that during cell division and subsequent cell expansion, the new primary wall is deposited, and the ancestral walls are stretched around the new cells. Throughout this process, pectic and other amorphous substances (2) must be deposited within and around the expanded ancestral walls and to a lesser extent the new primary wall. Although the current study has furnished no new information concerning the composition of the amorphous substances in the middle lamella, it has verified the ability of acidic substances and multivalent cations to stabilize this region. In addition, it has shown that the middle lamella is not exclusively an isotropic, amorphous, adhesive layer but is a complex structure which can contain ancestral walls, as well as cellulosic and noncellulosic intercell-wall fibrils.

ASPECTS OF CELL AND CELL WALL DEVELOPMENT

Differences in the wall ultrastructure of the epithelial, intermediate, and outer cells are described in the section "Structure of the Canal Complex." By studying cell wall ultrastructure, it was possible to trace the developmental history of the mature canal complex cells. Development of the three cell types in the mature canal complex in terms of ancestral walls and the direction and order of cell division is illustrated in Fig. 25. This figure is based on a large number of shadowed, ultrathin longitudinal and cross sections, and surface replicas. The study is incomplete, however, as it was made only in conjunction with the major aims of the thesis. Mahmood (55) and Bailey [page 30 of (31)] showed that at least four cambial daughter cells are enclosed by the cambial cell wall. This was partially verified in the current study and that of Dunning (13), where multilayered membranes were often observed where they crossed longitudinal wall interfaces. Therefore, in analyzing Fig. 25, it should be kept in mind that an additional wall may be present on the outer surface of all cells except the cambial cell. Cell diameters are schematic and related to the number of cell divisions. Relative dimensions are illustrated in Fig. 2.

Two methods of intermediate cell formation are shown in Fig. 25. In the first, (A), longitudinal division occurs before cross wall formation, while in the second, (B), it occurs only in a few cells and after cross wall formation. Formation by the second method definitely occurs, and mature cells which developed in this manner are visible in Fig. 7. The cell doublets in Fig. 23 probably illustrate this type of formation, also. However, only scattered examples of intermediate cell doublets were observed in the longitudinal ultrathin sections. This is confirmed by Bannan (28) who studied the development of resin canal tissue in the cambial zone of a number of conifer genera. He concluded, with reference to several Pinus species,

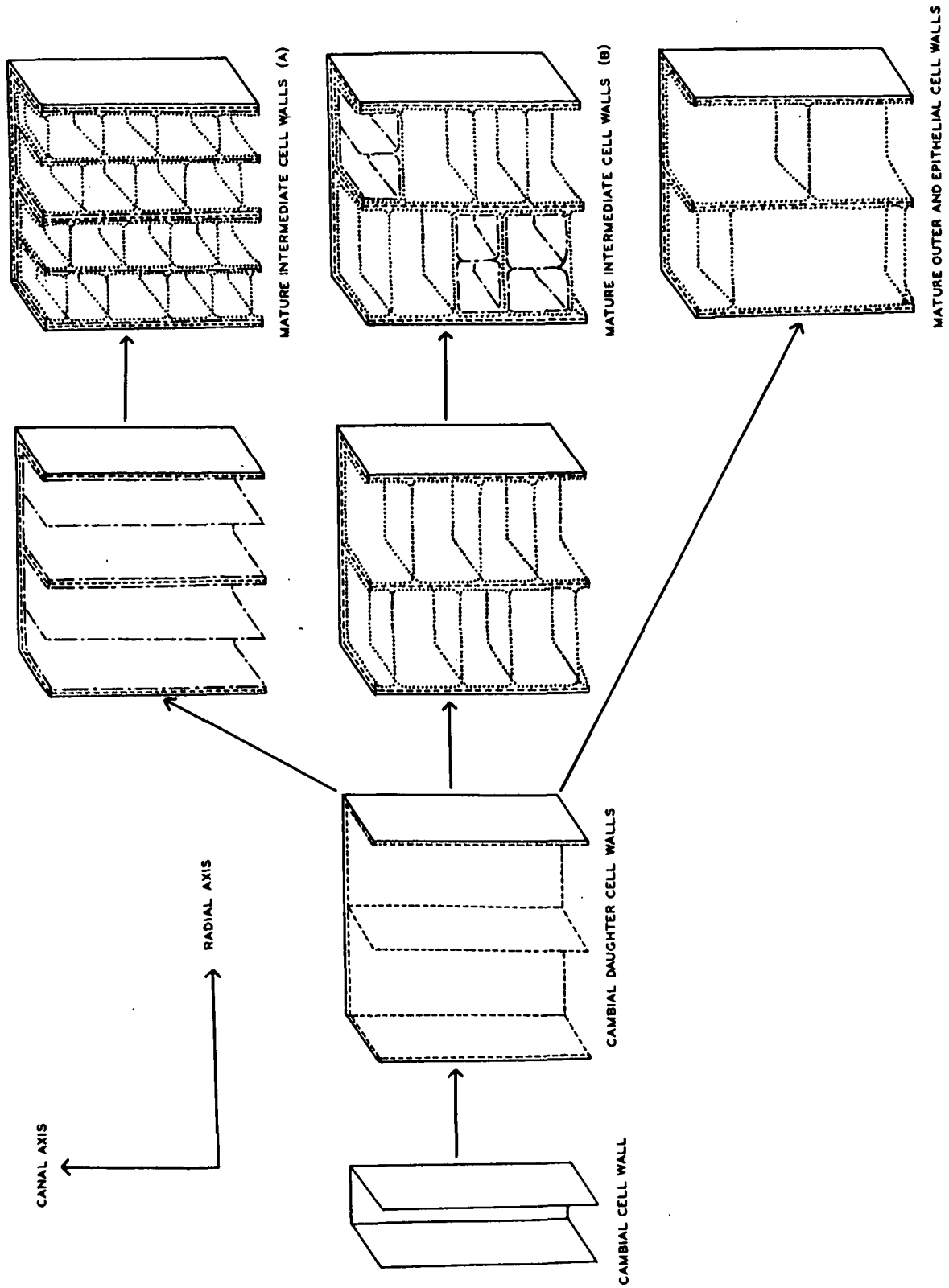


Figure 25. A Schematic Outline of Cell Wall Development in the Canal Complex (Drawn by Olga A. Smith)

that when longitudinal division occurs after septation, it is scattered and sporadic. Consequently, the first method of formation (A) is proposed to explain the common occurrence of doublets in the ultrathin cross sections and the smaller size of the intermediate cells (Fig. 2). Intermediate cell formation by this method requires that the (apparent) primary walls of the doublets in Fig. 23 are actually two walls; the primary wall deposited during cross wall formation and an ancestral wall associated with longitudinal division of the cambial daughter cells. The thick (apparent) primary walls of the doublets support this conclusion, particularly as little cell expansion would occur during cross wall formation (A). The encapsulating, multilayered ancestral wall structure surrounding the doublets would inhibit cell expansion, and the boundary between the two walls would not be apparent. However, the uncommon occurrence of doublets in the longitudinal sections could also be due to orientation of the partition wall (radial, tangential, or skew) because most sections were cut through the canal. Isolated instances of all three partition types were observed in the longitudinal sections. Since most of the partition walls observed in cross sections were directed along the radius of the canal (Fig. 23), they were not observed in the radially cut longitudinal sections. The above discussion shows that cell wall ultrastructure can be used to trace the developmental history of a mature plant tissue. However, further study is necessary to verify or disprove the existence of intermediate cell type A.

Although epithelial and outer cells are drawn to be identical in Fig. 25, they do differ in length, diameter, and wall thickness (Fig. 2). This is due to canal formation which determines the morphology of mature epithelial cells. Epithelial cells are characterized by a thin wall adjacent to the canal (Fig. 8 and 24) and a large diameter (Fig. 2). The existence of ancestral walls indicated by the intercellular membrane shown in Fig. 24 made it possible to trace the development of these cells and the canal. The canal is formed after the cell division which

produces the epithelial cells is complete. Adjacent epithelial cells are forced apart and separate along their radial interfaces, but at least some of the ancestral walls remain intact. The thin wall adjacent to the canal, and the large diameter of epithelial cells in the mature tissue, are attributed to wall stretching and subsequent cell expansion during separation. This mode of development is illustrated schematically in Fig. 26 and agrees with the schizogenous method of canal formation proposed by Bannan (28). Bannan studied the development of resin canal tissue in the cambial zone of several conifer genera and concluded that separation of adjacent epithelial cells was due to an accumulation of resin in the intercellular region.

A uniseriate ray associated with a mature canal complex is illustrated in Fig. 27. The intact and ruptured intercellular membranes (M) may form a continuous ancestral wall around the four ray cells (R) and the outer cell (O) on the canal complex surface (CS). The lack of continuity between the surface (CS) of this outer cell and the uniseriate ray is probably due to tissue distortion. If the encapsulating layer is a real structure, it is hard to explain in terms of current concepts of ray tissue development (31). Such a layer would necessarily need to be at least the wall skeleton of the original fusiform cambial initial. This is thought to be impossible as the wall would be ruptured during tissue development and maturation. Figure 27 is only included in the thesis because Dunning (13) observed similar structures surrounding mature tracheids and adjacent rays. Although the phenomenon is not considered further, the above discussion may initiate further work in this field.

INTERCELLULAR MEMBRANES AND ANCESTRAL PRIMARY WALLS

There is no doubt that at least remnants of ancestral walls exist on the outer surface of many primary cell walls in mature plant tissues. Intercellular membranes on, or associated with, primary cell wall surfaces have been observed where they

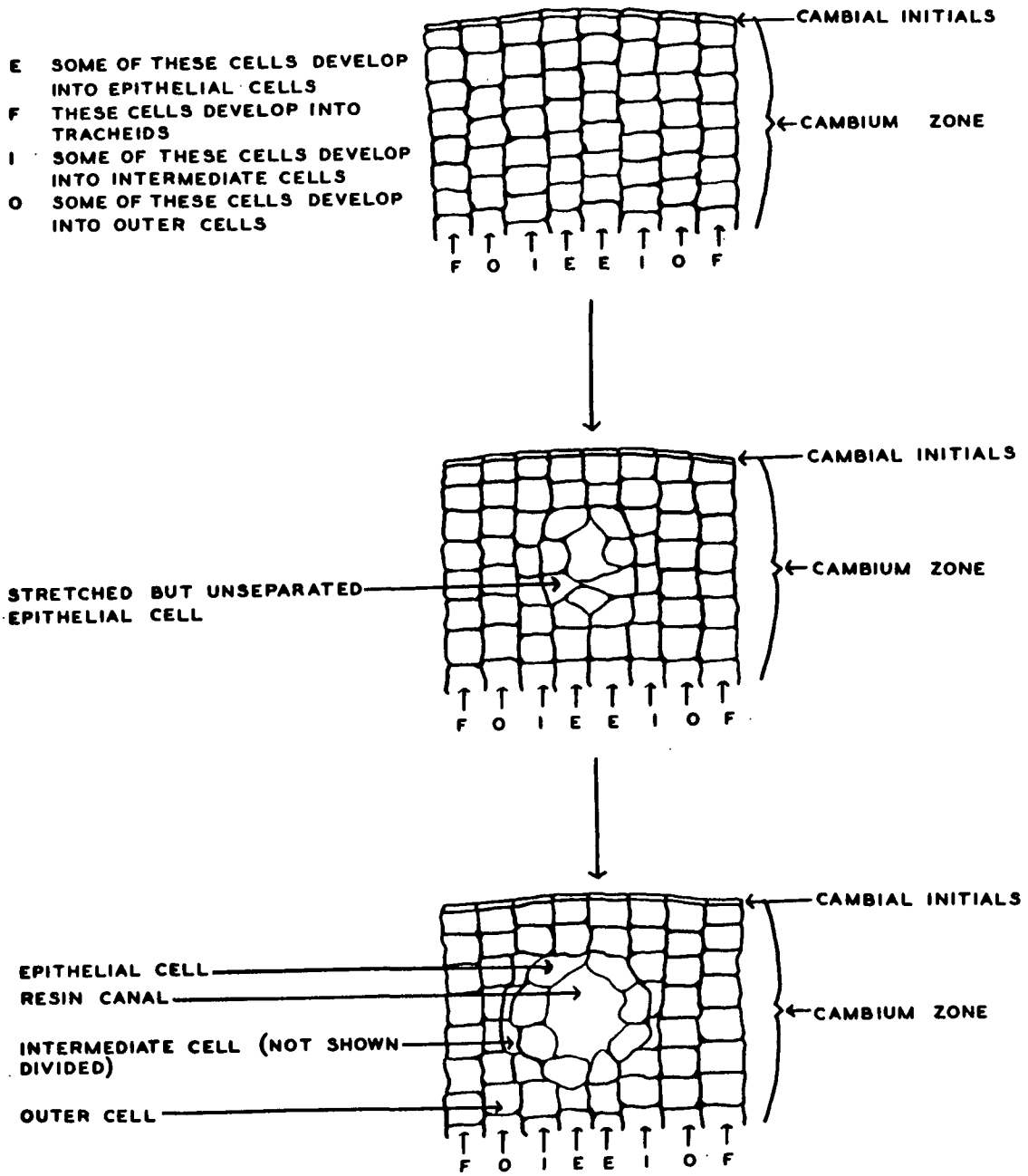


Figure 26. A Schematic Outline of Resin Canal Development, a Cross Sectional View (Drawn by Olga A. Smith)

Figure 27. Shadowed, Ultrathin Longitudinal Section of a Treated Canal Complex Showing Ray Tissue in Tangential Section

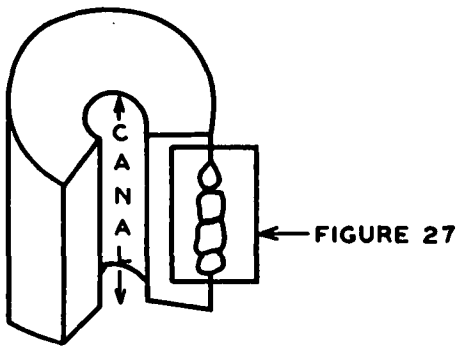
A: Canal complex axis
C: Cytoplasmic remnant
CS: Canal complex surface
M: Intercellular membrane
O: Outer cell
PF: Pit field
R: Ray cell

Plate Number: 6905AF

Magnification: 3900X

Tissue Treatment: Soaked in hydrochloric acid for 6 hours and washed.

Relation to canal complex:




O
I
A
M
R
M
PF
M
CS
O
R
C
M
R
M
R
O
4 µm
M

cross intercellular interfaces (13, 31, 55-57). Dunning (13) reviewed the work of Bailey [page 30 of (31)], Wardrop (56), and Newman (57) who observed such structures in sectioned cambial tissue with the light microscope. Jayme and Fengel (58, 59) observed with the electron microscope what were probably intercellular membranes between adjacent, delignified sprucewood tracheids. Mahmood (55), in a more recent publication, illustrated with light and electron micrographs the presence of membranelike structures which cover the radial surfaces of up to four cambial daughter cells. Because these structures exhibit positive birefringence under plane-polarized light, Mahmood concluded that they were ancestral walls. In addition, on the basis of the middle lamella thickness between adjoining tangential wall surfaces, he predicted the number of ancestral walls in these regions. Dunning (13) worked with mature, delignified, latewood longleaf pine tracheids and showed intercellular membranes in ultrathin cross sections and in replicas of the radial wall surfaces. These membranes appeared to be continuous over the radial surfaces of the tracheids, were often seen to be multilayered, and bridged the tangential interface between adjacent fibers. Although Dunning illustrated that intercellular membranes bridged the tangential interfaces of at least eleven consecutive tracheids, he was unable to show that these structures were connected and, therefore, continuous. However, Dunning's electron micrographs showed that the membranes are in fact typical of primary cell walls (1-5) because the fibrils were clearly visible and more or less randomly directed. This showed that membranelike structures observed in cambial tissue (31, 55-57) are actually portions of ancestral walls which retain at least their cellulosic skeleton after maturation. Because of the stretching and wall expansion which occurs during tissue maturation, it is difficult to conceive that the intercellular membranes observed by Dunning represent the original cambial initial wall. It is more likely that they represent the ancestral walls of cambial daughter cells,

as such walls probably only surround a maximum of four cells (55). By applying the techniques employed in the current thesis, with those of Dunning (13), to immature and mature tissue, it should be possible to characterize cell wall development and structure in wood. In particular, such a study would characterize the relation between cambial initials, the cambial zone (55), and the mature xylem.

Throughout previous sections of the thesis, the terms intercellular membrane and/or ancestral wall have been used in relation to the middle lamella, intercellular adhesion, and cell and cell wall development. It has been shown that the middle lamella can contain the cellulosic skeleton of multilayered ancestral walls which remain intact throughout cell maturation (Fig. 23). Adjacent ancestral walls, adjacent cell walls, and the ancestral wall adjacent to the primary wall are apparently connected by fibrillar and polymeric substances. Most of the amorphous substances must penetrate the ancestral walls as they are expanded and as the new primary wall is deposited. The ability of intercellular membranes (ancestral walls) to inhibit cell separation is particularly evident in canal complexes treated with potassium ferrocyanide and subsequently washed in water. Adjoining cell walls and wall elements (ancestral walls, lamellae, and fibrils) are separated by this treatment, but tissue identity is retained in the absence of mechanical stress. Figures 10, 19, and 23 show that ancestral walls and intercell-wall fibrils prevent complete tissue disintegration. The developmental history of the canal complex and the three cell types (Fig. 2) is based on the number of ancestral walls associated with each cell (Fig. 23 and 25). The remainder of this section illustrates more clearly the reasons for the above conclusions.

The ease and direction of cell separation in washed, potassium ferrocyanide or hydrochloric acid treated tissue are giverned by the number of ancestral walls surrounding adjacent cells. A number of ancestral walls (intercellular membranes) are visible in Fig. 10a and 28. The three electron micrographs overlap slightly

Figure 28. Shadowed, Ultrathin Cross Section of a Treated Canal Complex Showing a Number of Multilayered, Intercellular Membranes Surrounding Adjacent Intermediate Cells

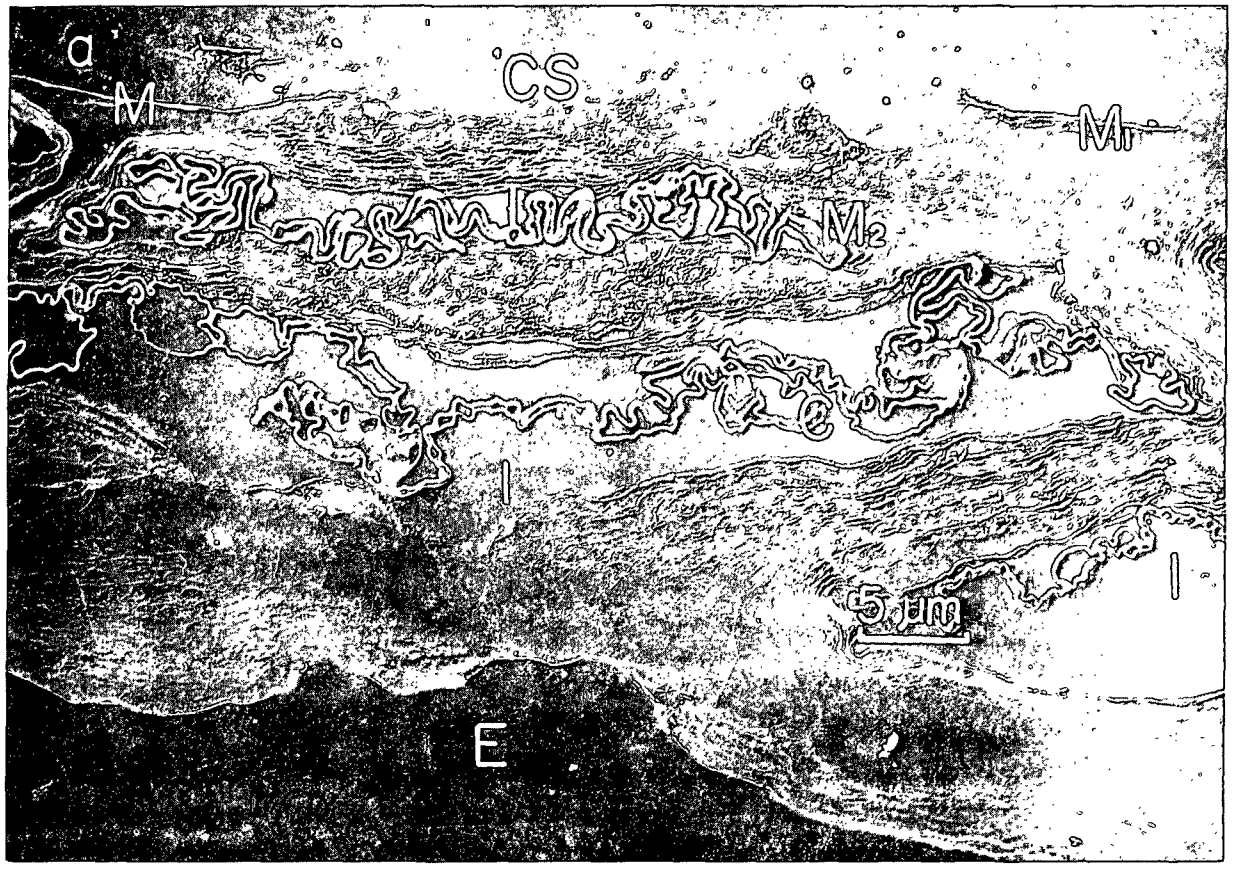
C: Cytoplasmic remnant
E: Epithial cell
I: Intermediate cell
CS: Canal complex surface
F: Intercell-wall fibrils
M:, M_1:, M_2:, M_3: Intercellular membranes

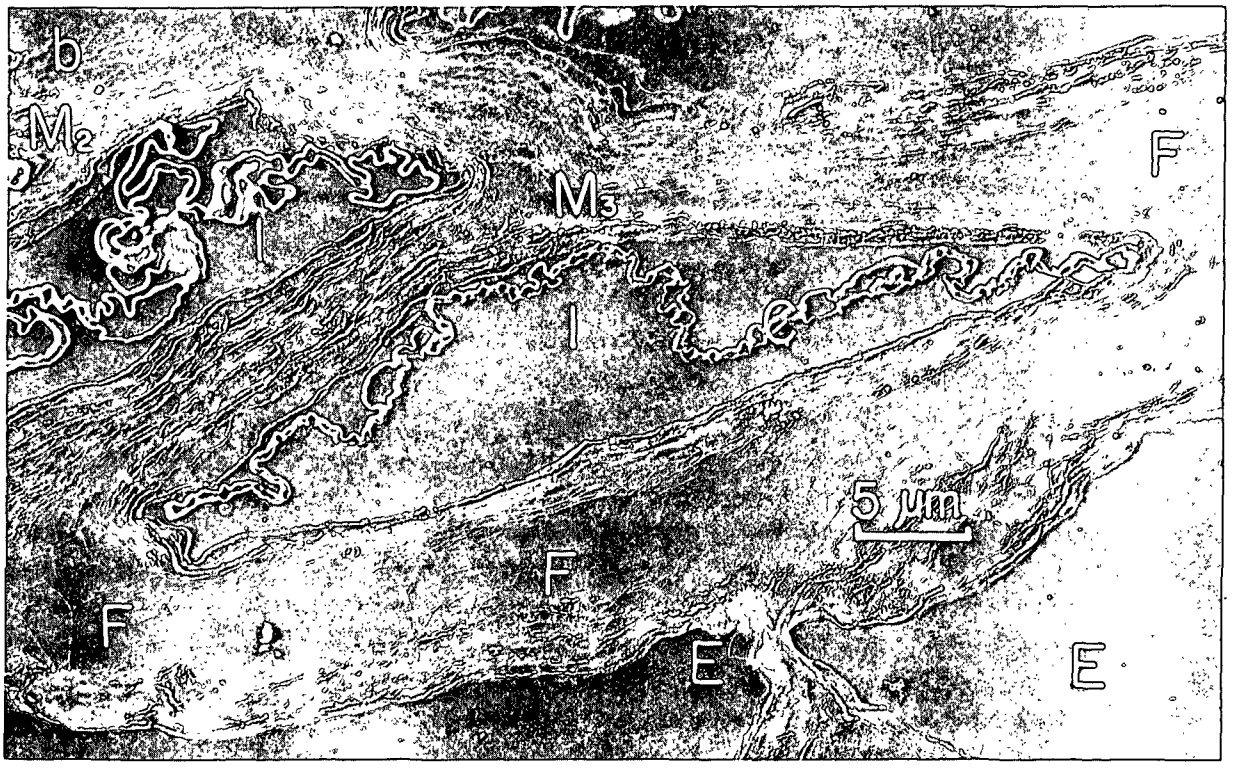
Plate Numbers: a. 7019AF
b. 6748AF

Magnification: 3000X

Tissue Treatment: Soaked in hydrochloric acid for 36 hours and washed.

See also Fig. 10a.


a
CS
M
M1
M2
I
I
5 μm
E


b
M2
M3
I
I
F
5 μm
F
F
E
E

and show an extensive area within a canal complex. Membrane M in Fig. 10a and 28a represents an ancestral wall on the canal complex surface which may be continuous with membrane M_1. The possibility that membrane M_1 is a wall remnant of a cell removed during canal complex isolation is not in accordance with the canal tissue as seen in surface replicas. Therefore, for the remainder of this discussion it is assumed that M and M_1 are portions of ancestral wall(s) associated with canal complex cells. However, membranes M and M_1 may not be part of the same ancestral wall. Two other membranes, M_2 and M_3, which are located within the canal complex, are shown in Fig. 28. Membrane M_3 probably represents a single ancestral wall which also passes over the M_2 interface. Therefore, membrane M_2 consists of the ancestral wall M_3 and the ancestral wall created when the partition wall associated with M_2 was deposited. These three electron micrographs (Fig. 10a and 28) as well as those in Fig. 19, 23, 24, and 27, show how it was possible to trace the development of the three cell types in the canal complex by examining cell wall ultrastructure. However, it was not possible to determine the origin(s) of the ancestral wall(s) M and M_1 on the canal complex surface. This wall(s) probably contains remnants of the original cambial initial wall as well as cambial daughter cell walls (Fig. 25). The ability of intercellular membranes (ancestral walls) to inhibit cell separation after most of the acidic substances are removed is also illustrated in Fig. 28. The degree of cell separation decreases as the number of ancestral walls incorporated in the membranes increases. This is illustrated by the interface associated with membrane M_3, which is separated to a greater extent than M_2. These two interfaces are separated to a lesser degree than those associated with membranes M and M_1, and this is attributed to the strengthening effect of these membranes on membranes M_2 and M_3.

Replicas of the canal complex surface also furnished information on intercellular membranes and their role in intercellular adhesion. Elevated cross walls

and the location of longitudinal wall interfaces are shown in Fig. 29. Although elevated cross walls were discussed previously (page 50), it is important to note that they could not be identified after the canal complex was subjected to a 36-hour treatment in potassium ferrocyanide or hydrochloric acid and subsequently washed. Except for longitudinal walls developed after septation (intermediate cell B — Fig. 25), the locations of longitudinal interfaces are readily identified in the surface replicas. This is explained in terms of Fig. 25 which shows that cross walls are normally the last to be formed and are always surrounded by at least two ancestral walls. In contrast, most longitudinal wall interfaces are bridged by a single ancestral wall which is expanded to a greater degree than later formed walls during tissue maturation. Consequently, cross wall interfaces in surface replicas of treated canal complexes are masked by thick, multiwalled intercellular membranes and are not visible.

Perforated intercellular membranes which bridge longitudinal wall interfaces are seen in Fig. 13b and 30. Although many of the fibrils within the membranes appear to be directed across the interface, the randomly oriented fibrillar organization, typical of primary walls, is visible (1-5). The appearance of partially directed fibrils is probably due to cell expansion during tissue maturation (13) and to tissue swelling when canal complexes treated with potassium ferrocyanide are subsequently soaked in water. The perforated appearance of the membranes is probably due to cell expansion and to the removal of acidic substances. In addition, non-cellulosic fibrils destroyed by the treatments may have penetrated the membranes at these locations. Although the membranes in Fig. 13b and 30 appear to consist of a single ancestral wall, remnants of an additional layer are visible in Fig. 30a. Layer PL was partially pulled from the canal complex surface probably during the isolation process (Appendix II). This feature is accentuated in Fig. 30a as many of the amorphous substances were removed when the isolated tissue was treated.

Figure 29. Surface Replicas of an Untreated Canal Complex. Figure 29b is a Magnified Area of Fig. 29a

A: Canal complex axis
E: Elevated cross wall — note steplike arrangement of three cross walls in (a)
L: Location of a longitudinal wall interface
PL: Part of an ancestral wall partially pulled from the canal complex surface

Plate Numbers: a. 5450AF
b. 5451AF

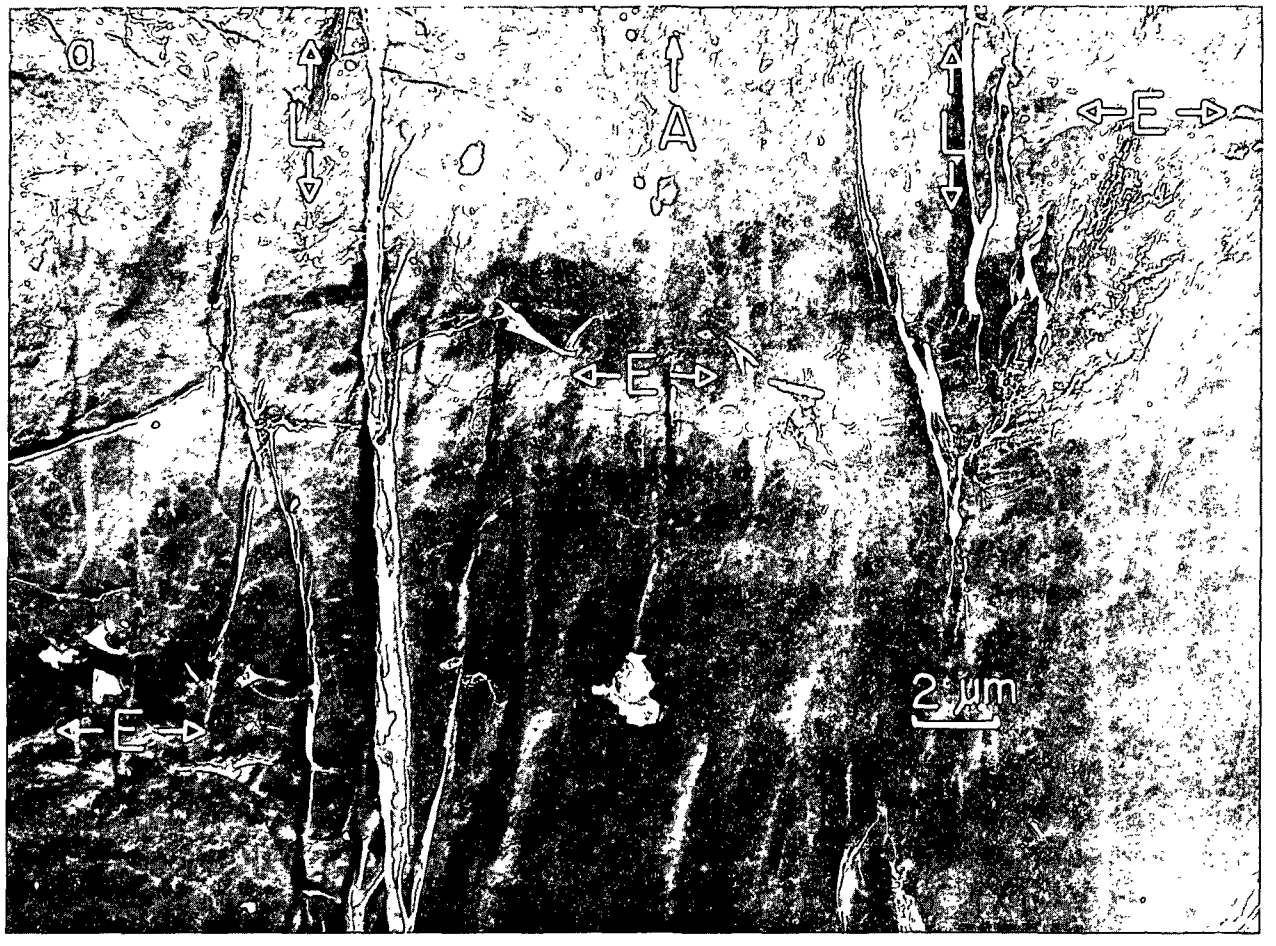
Magnification: a. 5700X
b. 11,400X

See also Fig. 12.

Examples of a layer (PL) and fibrils partially pulled from an untreated canal complex surface are shown in Fig. 29. The existence of wall layers partially separated from a canal complex surface does not necessarily indicate an additional ancestral wall because multilayered ancestral walls can exist (Fig. 23).

Longitudinal wall interfaces are readily identified in replicas of the canal complex surface as they are extremely long compared with cross walls and are normally bridged by a single, stretched ancestral wall (Fig. 25). The length of longitudinal wall interfaces is normally determined by the fusiform cambial initials. These statements refer only to longitudinal walls developed before septation (intermediate cell A — Fig. 25). Cross walls are small compared with the length of longitudinal wall interfaces (Fig. 2) and appear in a steplike arrangement when viewed in surface replicas (Fig. 29a).

Preferential cell separation along the longitudinal wall interfaces (Fig. 7) is apparently due to the differences between the longitudinal and cross walls. The thick, multiwalled intercellular membranes which bridge cross wall interfaces prevent


a
L
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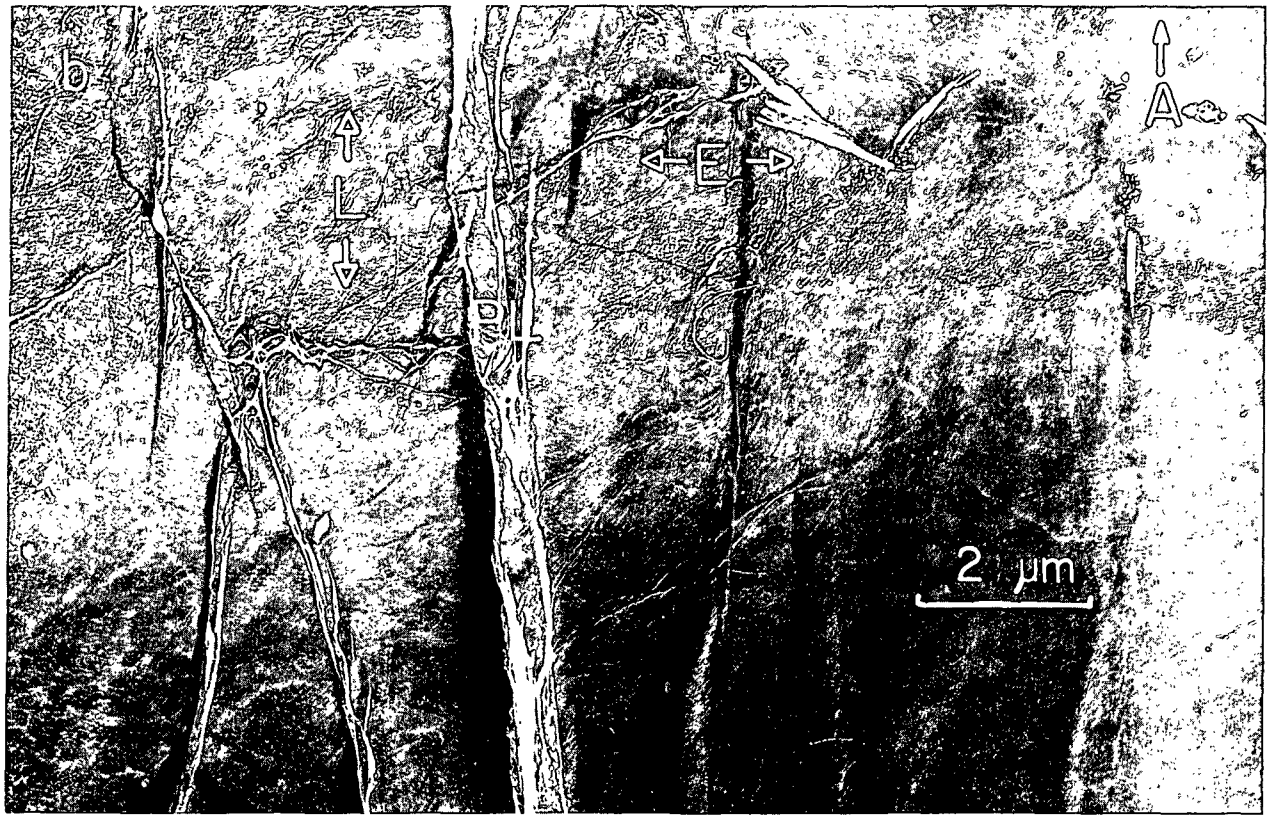

b
L
E
A
2 µm

Figure 30. Surface Replicas of Treated Canal Complexes Showing Perforated Intercellular Membranes Bridging the Longitudinal Wall Interfaces

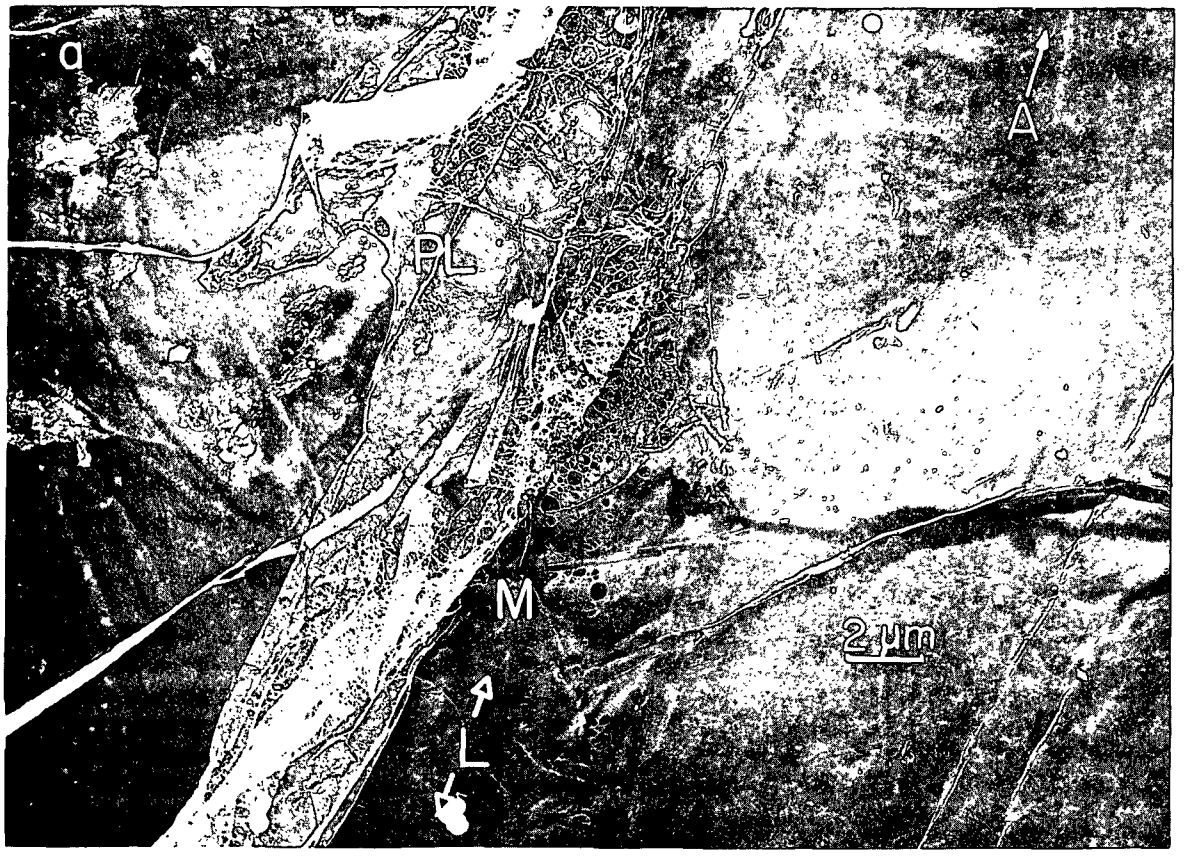
A: Canal complex axis
L: Location of a longitudinal wall interface
M: Intercellular membrane
PL: Part of an ancestral wall partially pulled from the canal complex surface

Plate Numbers: a. 6060AF
b. 5935AF

Magnification: a. 5300X
b. 15,000X

Tissue Treatment: a. Soaked in potassium ferrocyanide at pH 2.4 for 36 hours and washed.
b. Soaked in potassium ferrocyanide at pH 6.8 for 12 hours and washed.

cell separation but not wall separation. This is illustrated in Fig. 31 where adjacent cross walls (E) are separated but held together near the edges by the multilayered membranes M_1 and M_2. The remnants of at least six lamellae are visible in membrane M_2. Membrane M_1 probably represents a situation where cleavage occurred between ancestral walls of the adjacent cells rather than between the two cell walls (Fig. 24). Although cross walls are affected by the charge effect which develops when potassium ferrocyanide treated tissue is soaked in water, the effects of swelling are localized and resisted by the thick, multilayered intercellular membranes. Along the expansive longitudinal interfaces, tissue swelling has a much greater effect and tends to rupture the thin, restraining intercellular membranes. Fibril direction in the intercellular membranes may also affect the location of cell separation. Fibrils in the membranes which bridge the longitudinal wall interfaces tend to be directed across the gap (Fig. 13b and 30). Although this may increase separation between adjacent longitudinal walls, the effect is probably insignificant compared with the effect of swelling along the longitudinal interfacial


a
A
PL
M
L
2μm

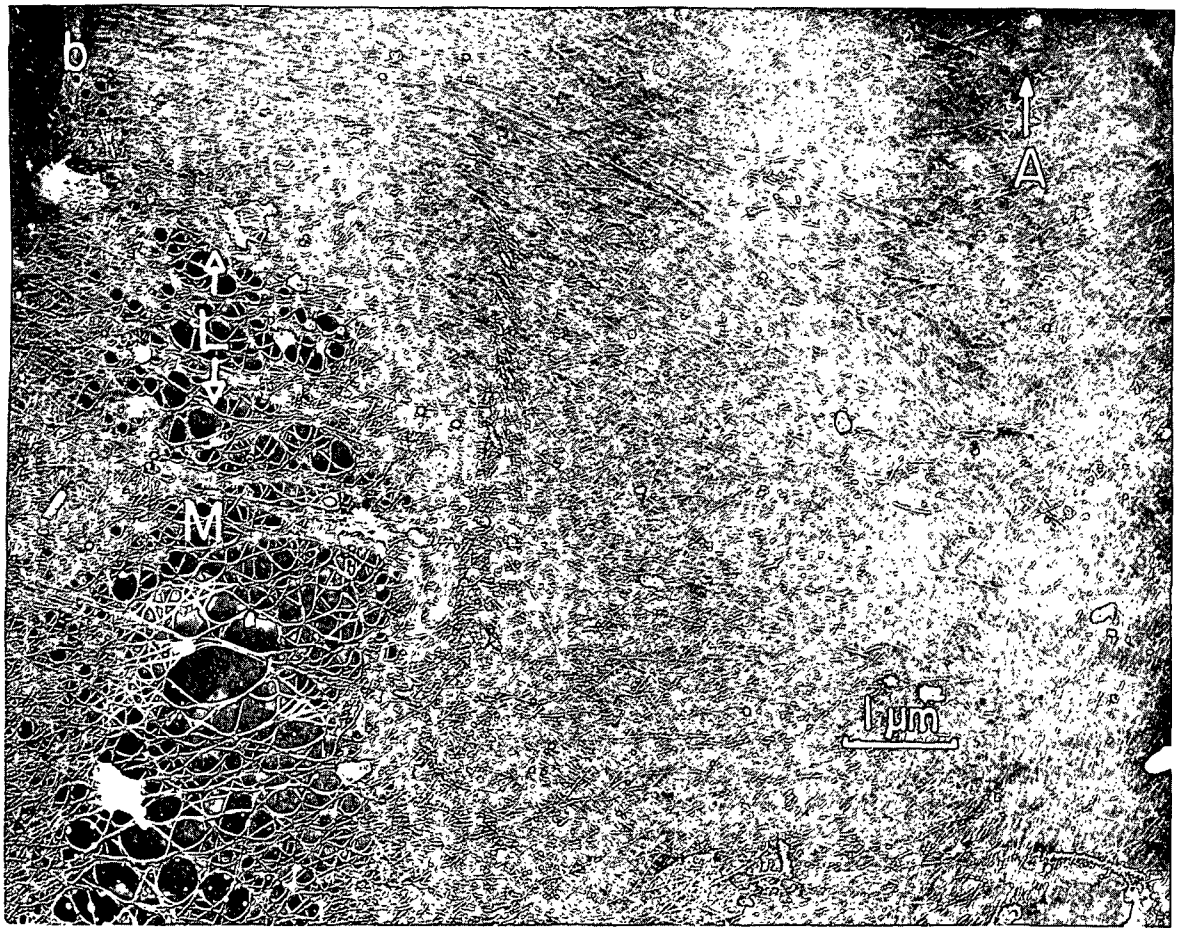

b
A
M
1μm

Figure 31. Shadowed, Ultrathin Longitudinal Sections of a Treated Canal Complex Showing the Ability of Intercellular Membranes to Prevent Cell Separation. Figure 31b is a Magnified Area of Fig. 31a

A: Canal complex axis
E: Cross walls
L: Longitudinal wall
M_1: } Intercellular membranes
M_2: }
O: Outer cell

Plate Numbers: a. 6780AF
b. 6781AF

Magnification: a. 3400X
b. 10,300X

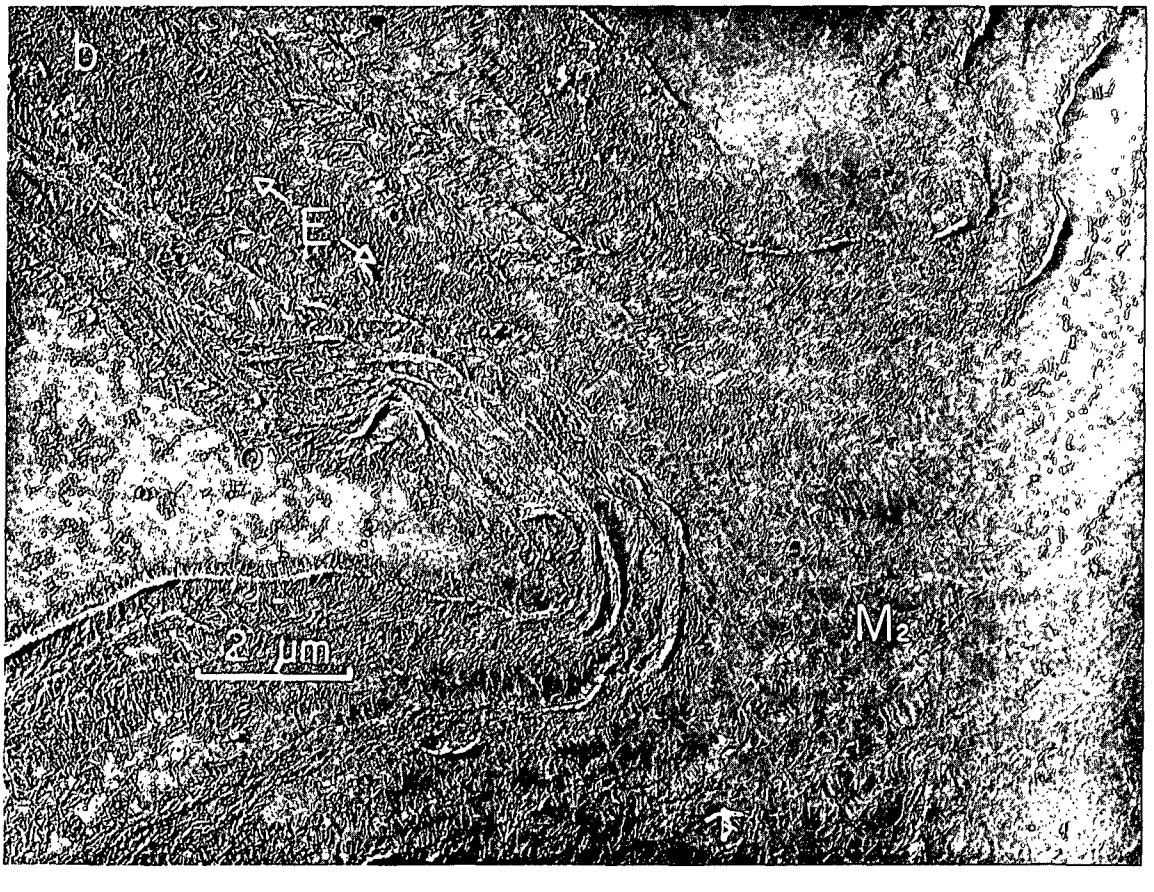
Tissue Treatment: Soaked in potassium ferrocyanide at pH 6.8 for 36 hours and washed.

regions. The hypothesis that different compositions of amorphous substances in the longitudinal and cross wall intercellular regions are associated with the variation in cell separation was rejected. The two regions appeared to be chemically similar in unstained, ultrathin sections (Appendix VI) and in shadowed, ultrathin sections of treated and untreated tissue.

INTERCELL-WALL FIBRILS

Current concepts concerning the middle lamella were reviewed in previous sections and showed, with the exception of Albersheim (5), that cellulosic fibrils are nonexistent in the true intercellular region. The term true intercellular region refers to the area between adjacent cell walls and not to the middle lamella. Although Albersheim had no data to indicate that cellulosic fibrils exist in the true intercellular region, he did not eliminate this as a possibility. Jayme and Fengel (58, 59) showed that in addition to intertracheid lamellae (membranes), scattered fibril-like structures, which appeared to connect adjacent fibers, were


a
M1
L
A
E
O
O
L
M2
5 µm


b
E
M2
2 µm

present in ultrathin sections of delignified sprucewood. However, these structures were apparently not observed in sufficient quantity or detail to enable the authors to determine whether they actually passed through the true intercellular region and connected adjacent tracheids.

The low magnification electron micrographs of intercell-wall fibrils in Fig. 10, 19, 23, and 28 show that these structures are not associated with intercellular membranes but actually pass through the true intercellular region and connect adjacent cell walls. In addition, adjacent lamellae, adjacent ancestral walls, and ancestral walls which adjoin primary walls all appear to be connected by fibrillar material. Although Jayme and Fengel (58, 59) and Frei and Preston (60) have shown that adjacent lamellae within plant cell walls are connected by fibrillar structures, the observation of distinct intercell-wall fibrils is unique to this thesis.

In preceding sections, intercell-wall fibrils were indicated in certain figures and their probable role in intercellular adhesion mentioned. However, the reader was obliged to accept the fact that fibrils which connect adjacent cell walls exist, so that the different phenomena in intercellular adhesion could be described. The following discussion will show that the intercell-wall fibrils indicated in Fig. 10, 19, 23, and 28 connect adjacent cell walls. The outer surfaces of adjacent, mature cell walls must be arranged according to one of the following alternative hypotheses.

1. Adjacent cell wall surfaces are separated by a thin, isotropic intercellular region which acts as an adhesive.

2. Many of the wall elements in adjacent cell wall surfaces are actually in contact (interwall) and are embedded in an amorphous matrix.

3. Adjacent cell wall surfaces are connected by cellulosic fibrils which pass through the true intercellular region. Therefore, this region contains fibrillar material embedded in an amorphous matrix.

The first hypothesis, which agrees with current concepts (1-5), is eliminated on the basis that adjacent cell walls would separate when most of the acidic substances are removed and when the canal complex is subjected to the charge effect which develops when the treated tissue is soaked in water. This conclusion is not affected by the presence of ancestral walls which prevent cell separation but not wall separation (Fig. 31).

If adjacent cell wall surfaces are in contact (Hypothesis 2), removal of the amorphous matrix should facilitate wall separation. Although approximately 80% of the acidic substances are removed, only small amounts of the hemicelluloses are extracted when the canal complex is treated with potassium ferrocyanide or hydrochloric acid (Table VI). This leaves the possibility that those wall elements which are in contact may be surrounded and held together by networks of the residual amorphous substances (page 48). However, where wall elements in adjacent cell wall surfaces are still in contact after treatment in potassium ferrocyanide, they should preferentially separate under the influence of the charge effect. Figure 10b clearly illustrates that this is not the case and that separation occurs throughout the middle lamella and the primary wall.

The third hypothesis concurs with the chemical data (Table VI) and the microscopic observations and could not be rejected. The absence of preferential separation in the true intercellular region and the presence of intercell-wall fibrils are illustrated in Fig. 32. Partial separation within the primary wall, rather than within the intercellular region, is seen in Fig. 32b. Differences in the appearance and thickness of intercell-wall fibrils and fibril aggregates between Fig. 10, 19, 23, 28, and 32 are due to different chemical treatments and magnifications. Intercell-wall fibrils and their relation to adjacent cell walls and wall lamellae are clearly visible in Fig. 33. Adjacent lamellae have short fibril remnants directed

Figure 32. Shadowed, Ultrathin Cross Sections of Treated Canal Complexes Showing Intercell-Wall Fibrils

E: Epithelial cell
F: Intercell-wall fibrils

Plate Numbers: a. 6755AF
b. 6791AF

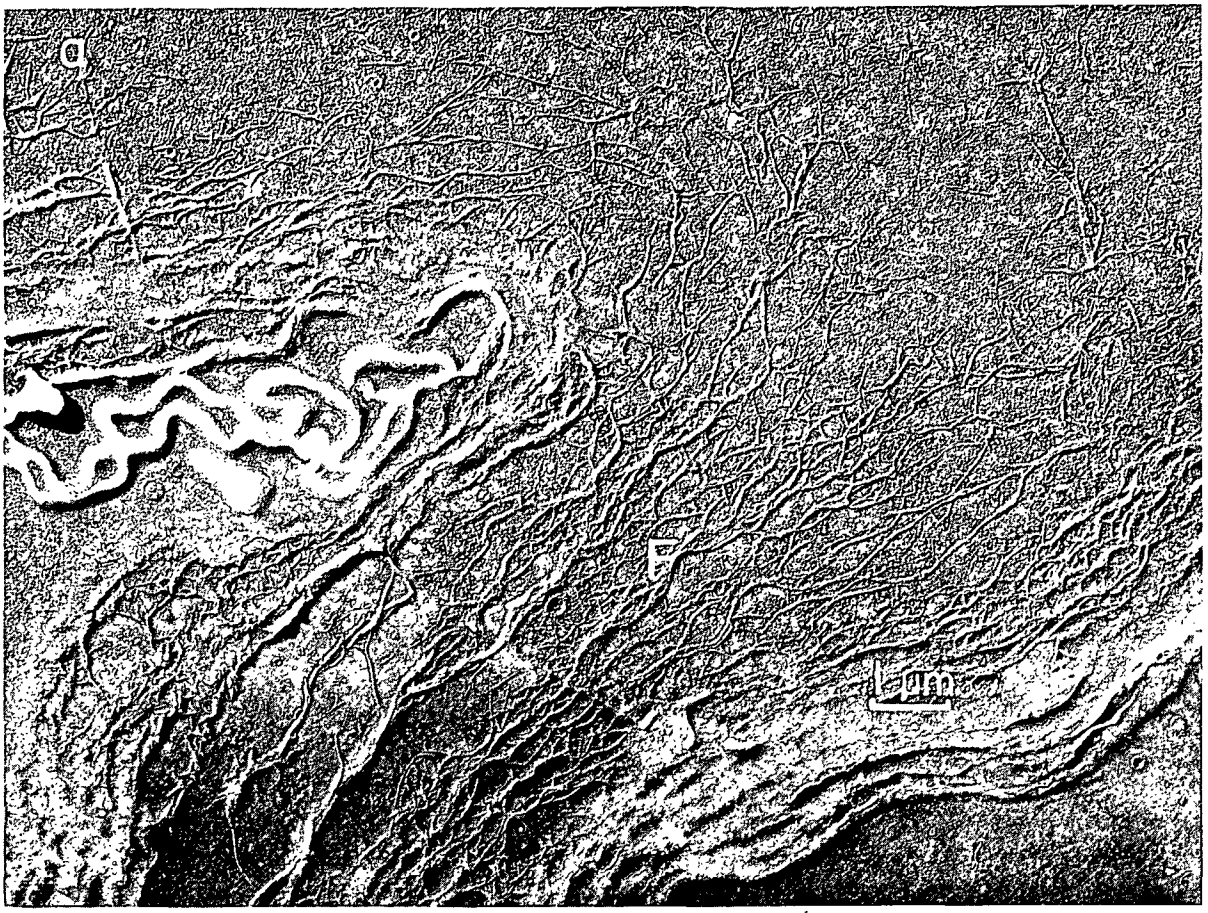
Magnification: 10,800X

Tissue Treatment: a. Soaked in hydrochloric acid for 36 hours and washed.
b. Soaked in potassium ferrocyanide at pH 6.8 for 36 hours, washed, soaked in ferric chloride at pH 1.9 for 6 hours, and washed again.

See also Fig. 10, 19, 23, and 28.

approximately parallel to one another and are separated by regions containing randomly directed fibrils. These fibrils appear to connect adjacent lamellae and adjacent cell walls. A cross-sectional view of such fibrils is shown in Fig. 32a. The two thick fibrils, F, are probably lamellae remnants, and the fibrillar network between them is equivalent to the randomly directed fibrils in Fig. 33. In addition, the area between the two fibrils is apparently the true intercellular region. The presence of intercell-wall areas which contain randomly directed fibrils explains why such regions do not exhibit birefringence under plane-polarized light, and, therefore, why the middle lamella is currently considered to be isotropic (1-5).

The canal complex is an excellent tissue with which to study intercell-wall fibrils as it has thin walls which are flexible under stress. Because of this flexibility, the cells are somewhat changed but retain their identity when treated, washed, and subsequently embedded. In addition, because intercellular membranes prevent cell separation but allow wall separation (Fig. 31), intercell-wall fibrils


a
F
1 µm

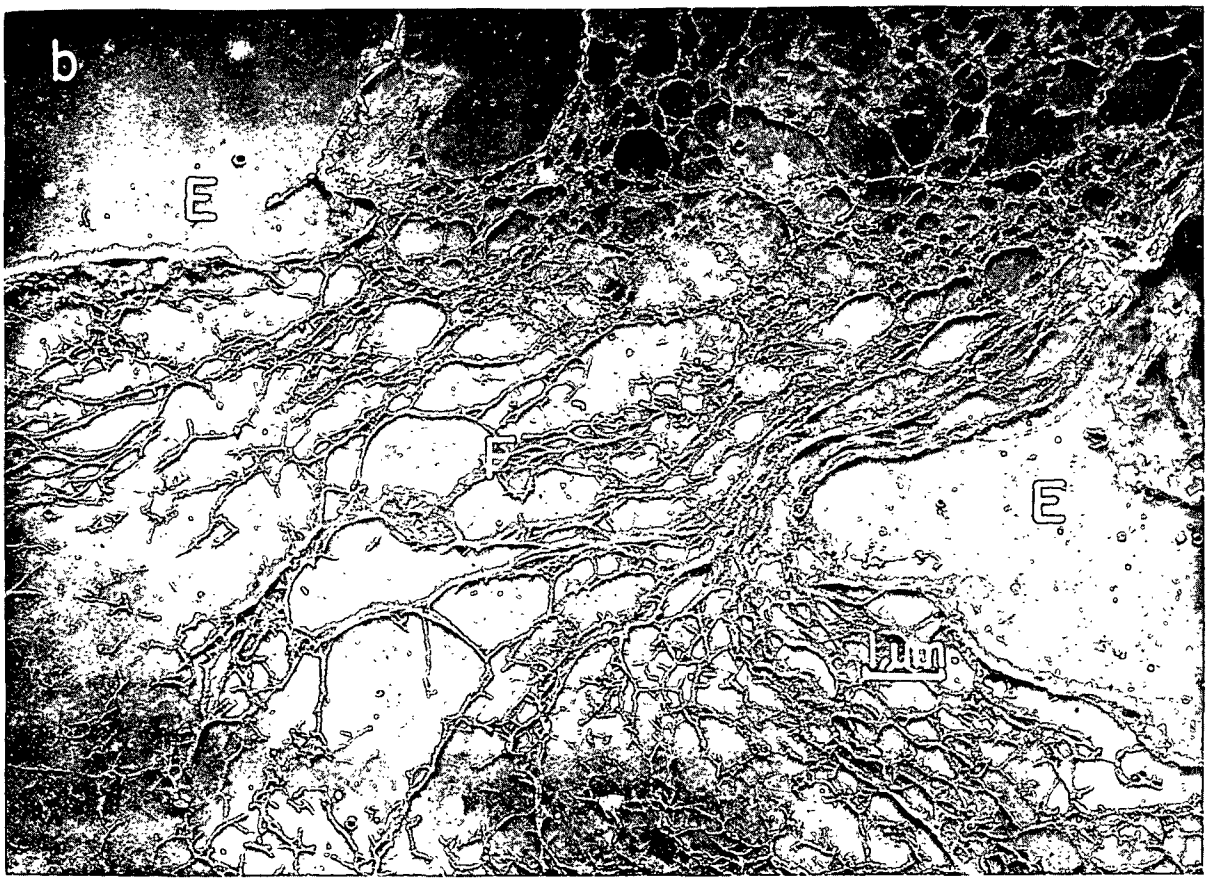

b
E
F
E
1 µm

Figure 33. Shadowed, Ultrathin Longitudinal Section of a Treated Canal Complex Showing Randomly Directed Fibrils Between Adjacent Wall Lamellae

A: Canal complex axis

Plate Number: 6843AF

Magnification: 22,000X

Tissue Treatment: Soaked in potassium ferrocyanide at pH 6.8 for 36 hours and washed.

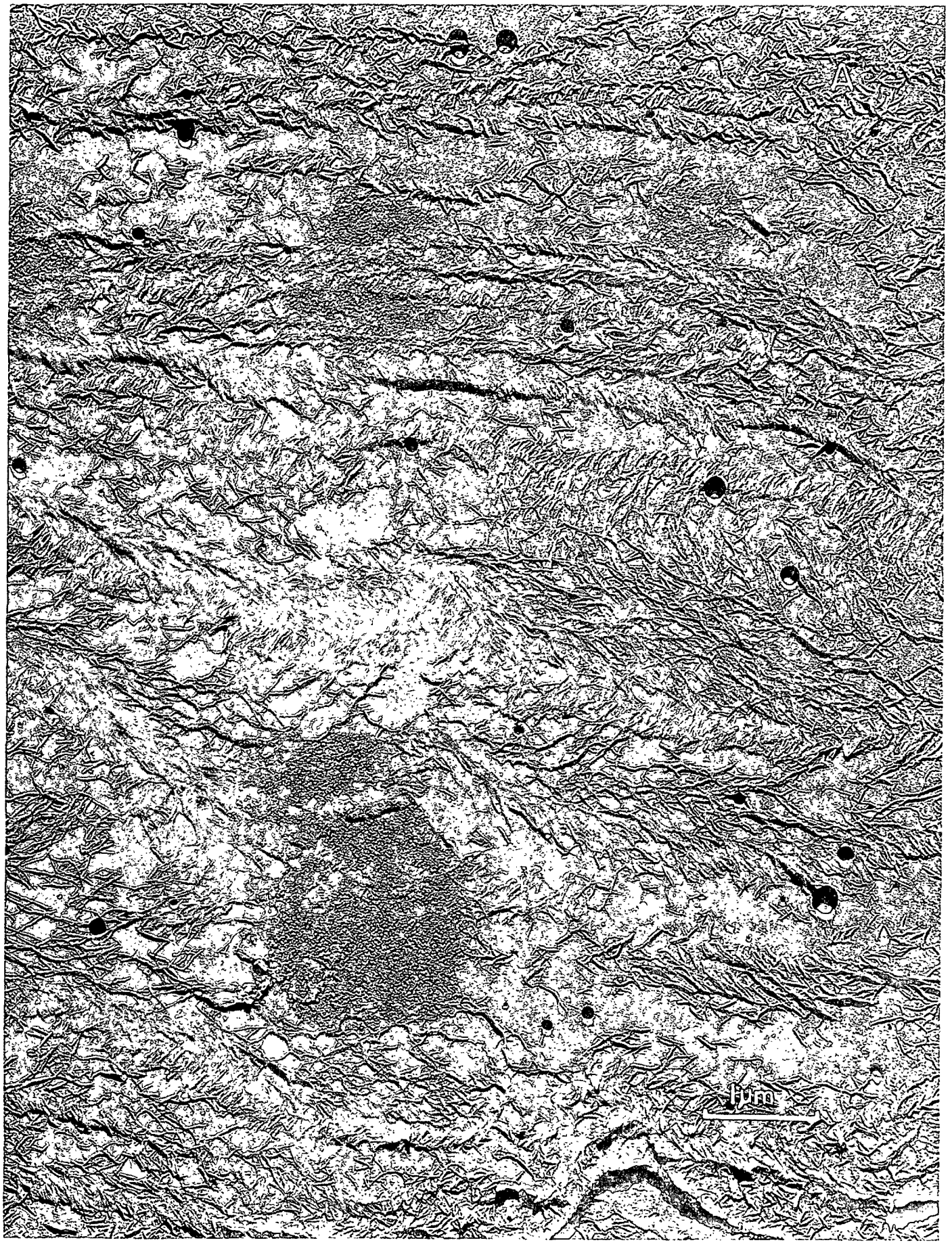
See also Fig. 21a.

remain intact, even when fragile canal complexes (potassium ferrocyanide treated, and washed) are prepared for observation with the electron microscope.

Although noncellulosic fibrils were only observed in surface replicas of the canal complex, they probably occur throughout the intercellular regions. These structures also probably connect adjacent cell walls and adjacent ancestral walls. Shadowed, ultrathin sections of the canal complex were examined in an attempt to identify noncellulosic fibrils in the middle lamella. Although fibrils which might be noncellulosic were observed, insufficient detail prevented positive identification. This was expected, as noncellulosic fibrils were only observed under very specific conditions in the surface replicas, and these could not be reproduced in the shadowed, ultrathin sections.

PIT FIELDS AND WALL PERFORATIONS

The term pit field rather than pit is used because the canal complex cells are without secondary thickening (3). Roelofsen (1), Esau (3), Frey-Wyssling and Mühlethaler (52), and Scott, et al. (61) discussed structural aspects of primary wall pit fields with particular reference to meristematic tissue and wall expansion during cell maturation. Although the structure of pit fields in the canal complex


A
1 µm

cells was not considered in detail in this study, it is interesting to note that their structure is similar but definitely not identical in appearance to that described in the literature (1, 3, 52, 61). Each pit field (Fig. 11b) contains many perforations which are organized in distinct clusters. This may be associated with the development of the canal tissue from the cambium rather than from apical meristems.

Although wall perforations in the canal complex occur in pit fields (Fig. 34) and in cross walls (Fig. 22), plasmodesmata are not visible. Plasmodesmata must be either destroyed during the chloriting procedure (Appendix I) or absent in the mature canal tissue. The presence of perforated pit fields and cross walls is in accordance with the observations of Livingston (62) who studied cells without secondary thickening from tobacco stems. Livingston observed plasmodesmata in pit fields and throughout the cross walls of these cells with a light microscope. In a more

Figure 34. a. Stained, Ultrathin Cross Section of an Untreated Canal Complex Showing Pit Fields

PF: Pit field

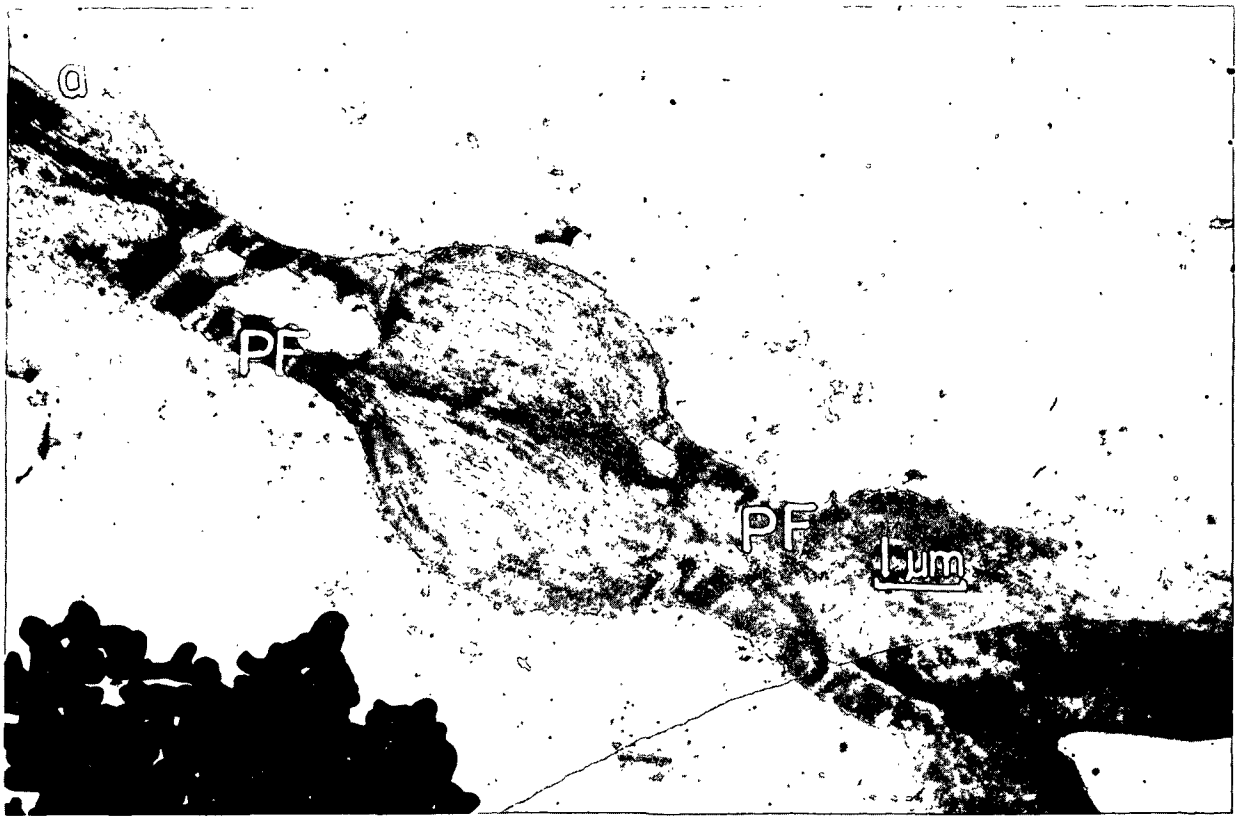
b. Photomicrograph of a Canal Complex Treated with Potassium Ferrocyanide and Separated by the Application of a Cover Slip. Pit Fields are Visible as Perforations or Holes in the Cell Walls

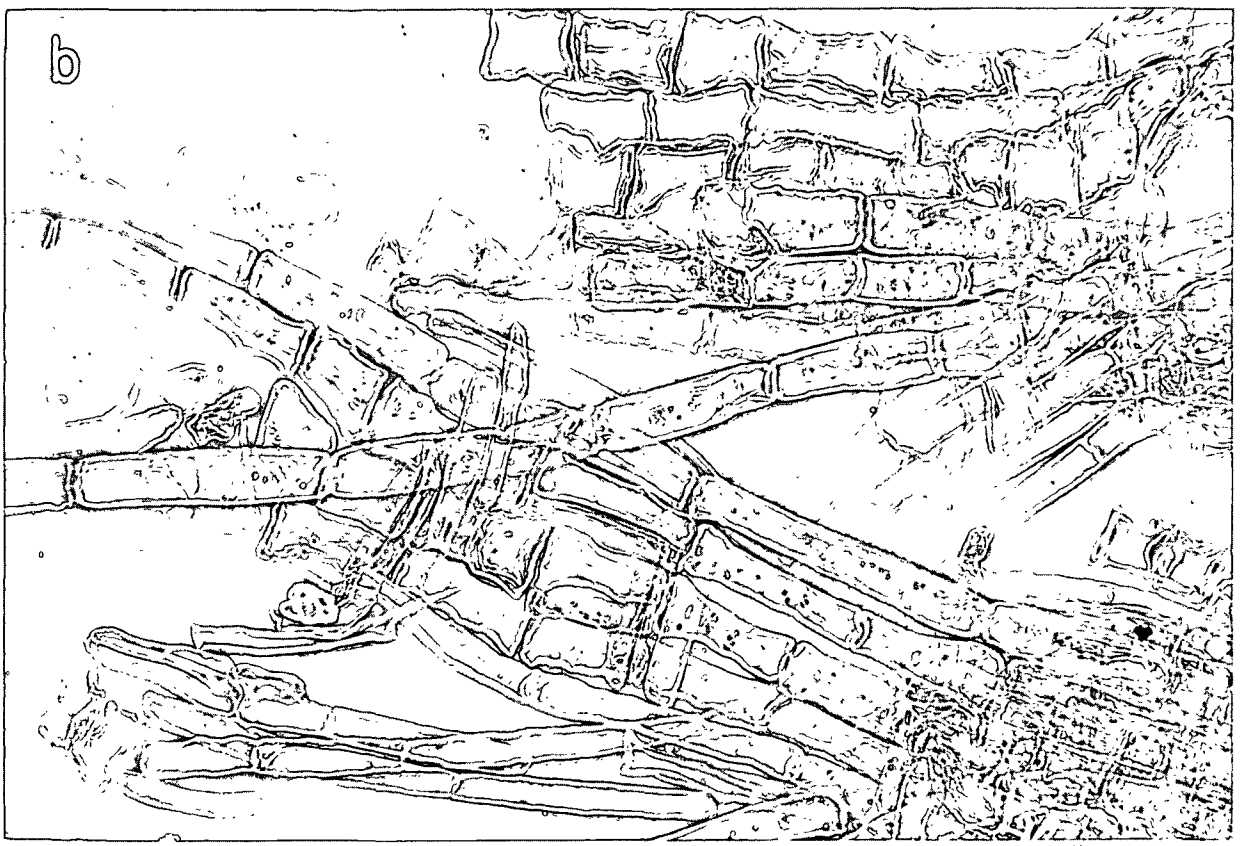
Plate Numbers: a. 6607F
b. 68M-656D

Magnification: a. 12,000X
b. 160X

Tissue Treatment: a. Untreated
b. Soaked in potassium ferrocyanide at pH 6.8 for 2 hours and washed.

See also Fig. 10a and 11.


a
PF
PF
1 µm


b

recent publication (63), Livingston describes plasmodesmata which are normally continuous through adjacent cross walls of mature resin canal cells (Pinus strobus). However, it is not known whether the plasmodesmata were observed in the epithelia or in the surrounding parenchyma cells or whether Livingston looked for pit fields in the longitudinal walls. Pit fields were observed only in longitudinal cell walls in the canal complex. It is apparent from the above discussion that the pit fields and cross wall perforations observed in the canal complex must represent the location of plasmodesmata destroyed during the chloriting process.

In both the canal complex and tobacco stem cells (62), wall perforations which are not associated with distinct pit fields are only observed in the cross walls. However, the indented appearance of pit fields may be associated with both ancestral walls on the longitudinal primary walls and with cell expansion during tissue maturation. Therefore, the statement of Livingston (63) that cross walls which contain plasmodesmata represent a single pit field is questionable.

Those portions of the primary cell wall and middle lamella that are associated with pit fields and wall perforations appeared to separate as readily as other wall areas. This was apparent in both untreated and treated canal complexes. Apart from the thin primary wall skeleton, the pit field indicated in Fig. 10b is similar to the adjacent wall structure. Although plasmodesmata are absent in the canal complex, the drawings of Livingston (63) indicate that they have an insignificant effect on cell adhesion.

THE CANAL COMPLEX AND DELIGNIFIED TRACHEIDS

It is interesting to compare the electron micrographs of Dunning (13), which show the radial walls of latewood, longleaf pine tracheids in surface view, with those of the canal complex surface. Replicas of both surfaces show intercellular

membranes (sometimes multilayered) which bridge longitudinal wall interfaces and contain fibrils that are often transversely directed. The cell wall surfaces are essentially identical with respect to fibril orientation and are typical of primary walls (1-5). These similarities are to be expected, as both tissues develop and differentiate in the cambial zone (31). The most noticeable differences between the two are the presence of pit fields in the canal complex cell walls, and pits in the tracheids. These differences are due to dissimilar tissue differentiation and to the effect of secondary wall development in the tracheids.

The primary walls of the canal complex contain fibrils which are organized in distinct lamellae and are directed approximately at right angles to the tissue axis (Fig. 21a and 33). The presence of distinctly lamellated and directed fibrils indicates that a secondary S_1 layer (13, 64) may be deposited on the inner surface of intermediate and outer canal complex cells. Wardrop (64) describes the primary walls of immature tracheids as containing transversely directed fibrils which are not arranged in distinct lamellae. However, because of the difficulties involved in defining the S_1-primary wall boundary (13), particularly in mature tissue, the canal complex cells are considered to have only primary walls (page 74).

CONCLUSIONS

The thesis was designed to characterize the middle lamella relative to boundaries, structure, and chemical composition, and to determine the effects of these criteria on intercellular adhesion. Emphasis was placed on structure, as definition of the middle lamella was considered essential before an understanding of intercellular adhesion could be reached.

The boundaries of the middle lamella are not smooth or definable but merge with the primary cell wall and are crossed by fibrillar and apparently polymeric substances. However, the locations of the boundaries are evident when the calcium pectate-rich middle lamella is stained or when the primary wall-ancestral wall interface is traced. Pectic substances are present in smaller quantities in the primary wall.

In contrast to current concepts, the middle lamella in the canal complex is not exclusively an isotropic, amorphous, adhesive region but is a complex structure which can contain ancestral cell walls as well as cellulosic and noncellulosic fibrils. Although calcium pectate is the major constituent of the middle lamella, other substances are present. Ancestral cell walls are composed of cellulosic and probably some noncellulosic fibrils and are embedded in the middle lamella as defined in this thesis. However, the intercellular region between adjacent cell walls also contains cellulose in the form of intercell-wall fibrils. The hemicelluloses are apparently closely associated with the cellulosic fibrils in the primary wall and the middle lamella. Although noncellulosic fibrils (probably polygalacturonan) were observed only in those parts of the middle lamella between the canal complex and adjacent tracheids, they probably also exist in intercellular regions within the canal complex. Substances in the canal complex (isolated from a chlorite holocellulose) which contain organic nitrogen are apparently not significantly associated with the middle lamella or intercellular adhesion.

Adjacent cell walls are connected by intercellular membranes and intercell-wall fibrils. Intercellular membranes have a very definite role in intercellular adhesion and prevent cell separation but not cell wall separation. This occurs after most of the pectic substances are removed and when the adjacent cells are subsequently subjected to stresses which pull them apart. Intercellular membranes are actually those parts of ancestral walls which bridge intercellular interfaces. Ancestral walls have apparently intact skeletons of cellulose fibrils and encapsulate cells developed within them. The greater the number of ancestral walls incorporated into an intercellular membrane, the greater the resistance to cell separation. In addition, the effectiveness of an intercellular membrane to resist cell separation is governed by the area of the interface it bridges. Intercell-wall fibrils, which are distinct from intercellular membranes, connect adjacent cell walls, adjacent primary walls, adjacent ancestral walls, adjacent wall lamellae, and adjacent ancestral and primary walls. These fibrils must have a significant effect on intercellular adhesion, particularly in reinforcing the calcium pectate matrix. In addition to cellulosic fibrils, the noncellulosic fibrils in the canal complex-tracheid interface also probably connect adjacent cell walls. Adjacent cell walls do not separate preferentially within the true intercellular region but along cleavage planes throughout the middle lamella, throughout the primary wall, and along the primary wall-middle lamella boundary. This is to be expected, as adjacent walls in the middle lamella and the primary wall are connected by fibrillar networks. Plasmodesmata and any associated cell wall or middle lamella structures have an insignificant role in intercellular adhesion.

Preferential cell separation in the direction of the canal complex axis is due to the thick, multilayered membranes which bridge the small cross wall intercellular regions and prevent separation at these locations. Normally, only single-layered membranes bridge the expansive longitudinal intercellular regions, and,

therefore, separation occurs preferentially at these interfaces. Because there is apparently no difference in the composition of the amorphous matrix in the cross and longitudinal intercellular regions, preferential separation between adjacent longitudinal walls is due to structural rather than chemical differences.

Bonding between multivalent cations and the acidic substances of the amorphous matrix in the canal complex was the only significant form of chemical bonding identified. However, the existence and significance of other forms of chemical and physical bonding were recognized but not studied extensively. In addition to bonding, the role of multivalent cations in intercellular adhesion is to stabilize and prevent solution of the acidic substances. Removal of the cations and most of the acidic substances does not cause cell separation unless the tissue is subsequently swollen by an aqueous medium of approximately zero ionic strength or subjected to mechanical stress. The retention of tissue identity after the multivalent cations and most of the acidic substances are removed is attributed to the influence of intercellular membranes and intercell-wall fibrils. These structures are apparently closely associated with the predominantly hemicellulosic, residual amorphous substances. When tissue from which the cations have been removed is swollen, adjacent wall elements are forced apart and any residual bonds and connecting polymeric networks are ruptured.

The calcium-stabilized pectic substances in the canal complex form an amorphous matrix which surrounds structural elements in the middle lamella and to a lesser extent in the primary cell wall. The bonding sites between calcium and the negative centers are randomly scattered throughout the amorphous matrix. Canal complex cells which are treated with potassium ferrocyanide, soaked in water, and manually separated, can be readhered by the addition of a solution containing multivalent cations, provided adjacent surfaces are in close proximity. The readhesion of separated cell wall surfaces is primarily due to chemical bonding rather than to flocculating forces.

GLOSSARY

Ancestral wall	A primary wall inside which a new wall has formed as a result of cell division.
Canal complex	The resin canal tissue isolated from a chlorite holocellulose which consists of a long tubular cavity surrounded successively by epithelial, intermediate, and normally, outer cells.
Cell wall	The composite wall which consists of the primary wall and any associated ancestral walls.
Epithelium	The single layer of epithelial cells which surrounds the cavity in the canal complex.
Fibril	A threadlike structure in the cell wall visible under the electron microscope. The term does not refer to a cellulosic elementary fibril which has a definite diameter (3.5-4.0 nm.).
Intercellular matrix	The amorphous substances of the middle lamella.
Intercellular membrane	A single or multilayered structure which passes over the interface between adjacent cells.
Intercell-wall fibril	A fibrillar structure which connects adjacent cell walls.
Intermediate cells	At least one complete layer of short, cubelike cells located adjacent to the outside surface of the epithelium in the canal complex.
Middle lamella	The intercellular region or layer between adjacent primary walls.
Outer cells	Elongated cells on the surface of the intermediate cell layer in the canal complex.
Primary wall	That structure which encloses a protoplast in the mature canal complex.
Pit field	A small, depressed area in the primary cell wall which contains multiple perforations.
Wall perforation	A small opening in the cell wall.

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APPENDIX I

PREPARATION OF THE CHLORITE HOLOCELLULOSE

A slash pinewood bolt (4 feet 6 inches long and 12 inches in diameter) was obtained from the Union Camp Corporation, Savannah, Georgia. This bolt was cut into disks 3/4 inch thick and subsequently into chips 3/4 inch by 1 inch by 1/4-1/2 inch. The oven-dried content of the chips was 57.2%.

The holocellulose was prepared by immersing chips in methanol for at least 2 days to remove moisture, then extracting with a chloroform-methanol mixture (1:1) for 3 weeks. The extracting liquor was replaced twice during this period. After extraction, the chips were soaked in methanol for at least 2 days to remove residual chloroform, dried under vacuum, soaked in water, and given a sodium chlorite treatment at room temperature (15, 16). Technical-grade sodium chlorite was added in one-pound lots to the treatment liquor, giving a 20-25% concentration based on dry wood. The pH was maintained between 4 and 5 by adding suitable quantities of glacial acetic acid. Further sodium chlorite was added in one-pound lots when the oxidant was consumed. Reaction was terminated after one month when the canal tissue could be isolated by the methods described in Appendix II. Approximately one pound of sodium chlorite was required for every 250 grams of wood treated (dry weight). The delignified chips were filtered and washed to remove residual chemical and frozen in polyethylene bags. Washing consisted of soaking the chips in water that was changed daily for 3 to 5 days. The first wash contained calcium acetate at 38 g./liter to strengthen the resin canal tissue (16).

APPENDIX II

ISOLATION AND PURIFICATION OF THE CANAL COMPLEX

The pulp was thawed and refrozen at least three times. This decreased adhesion between tracheids and the surface cells of the canal tissue apparently without affecting the cellular structure. In the isolation procedure, the pulp was thawed, defibered with a laboratory stirrer at a suitable consistency ($\approx$ 1%), and subjected to treatment with the following apparatus. A globular-shaped nichrome wire framework (diameter approximately 3 inches), consisting of numerous vertical and oblique supports, was attached to the rod of a laboratory stirrer. This framework, when rotated in the slurry, preferentially collected the long and flexible canal complexes. After 2-4 minutes rotation in the slurry, the framework was dipped in a beaker containing only water which washed away many of the fibers collected with the canal tissue. The canal complexes were then washed and/or picked off the framework. This process was repeated until most of the canal tissue was removed from the pulp. The addition of calcium to the holocellulose strengthened the canal tissue and was an essential step in the isolation procedure. In order to remove further fibrous debris, a hooked nichrome wire (16) was rotated in a very dilute slurry of the isolated material and preferentially collected the canal complexes. After repeating this procedure a number of times, the only remaining impurities were the tracheid and ray cells actually attached to the canal tissue. The isolated canal complexes were stored at 4°C. in 60% ethanol.

The purification procedure used by Thompson (16) was not suitable for tissue isolated by the above method as the calcium pretreatment apparently prevented separation of the fiber remnants and the canal complexes during hot water extraction. However, practically all fibrous material was removed if the tissue was subjected to a mild sequestering treatment prior to extraction, but it was not

possible to remove all ray cells because of their close association with the canal tissue during development and growth (page 85). The following purification procedure was employed. Five milliliters of 0.1% sodium hexametaphosphate was added to approximately 80 ml. of a dilute slurry of the canal tissue. This mixture was shaken violently for 1.5 minutes and poured into 600-700 ml. of gently agitated water for 2-4 minutes. The canal tissue was then transferred by a hooked wire to water at 55-65°C. and agitated for one hour.

APPENDIX III

ANALYTICAL PROCEDURES USED IN THE CHEMICAL COMPOSITION STUDIES

Acetyl — Whistler and Jeanes (65).

Ash — TAPPI method T 211 m-58 modified to 475-500°C.

Emission spectroscopy — Piper and Borchardt (66).

Galacturonic polyuronides — McCready and McCromb (67), except that chromatographic separation of galacturonic acid was used (66, 68).

Klason lignin — TAPPI method T 222 m-54.

Methoxyl — TAPPI method T 2 m-60.

Organic nitrogen — Henwood and Garey (69).

Polyuronides — Browning (70).

Sugars — Gas chromatographic method of Piper and Borchardt (66).

APPENDIX IV

LIGHT MICROSCOPY

Light microscopy was used to study the canal complex in untreated wood and wood soaked in acidified sodium chlorite for 10 and 32 days. The latter two samples received a three-week extraction treatment (Appendix I). The holocellulose (32-day treatment) was unsuitable for sectioning because the paraffin embedding medium caused distortion and collapse of the canal tissue. Attempts to prepare sections from the holocellulose chips were discontinued as cross sections of the canal complex embedded in maraglas (Appendix V) showed no tissue distortion. Cross and longitudinal sections of canal complexes embedded in maraglas were cut 1-5 μm. thick with a glass knife on a Porter-Blum ultramicrotome. Photomicrographs were taken with a Zeiss photomicroscope using 35 mm. Panatomic-X Kodak film.

Photomicrographs of cross, radial, and tangential sections were prepared from the untreated and partly treated wood samples. A number of half-inch cubes were prepared from each sample. The cubes were boiled in water to remove air, sectioned (25-30 μm. thick) on a sliding microtome, and stained with a phloroglucinol-hydrochloric acid mixture or Sudan IV (23). Sections stained in Sudan IV were rinsed quickly in 70% alcohol before mounting in a glycerin-water mixture. The remaining sections were stained with Heidenhain's iron alum haematoxylin and Safranin O (23), dehydrated in increasing percentages of alcohol, cleared in xylene, and mounted in Canada Balsam. Because no softening or embedding techniques were employed in preparing the wood samples for sectioning, thicker-than-normal sections were cut to prevent tearing of the canal tissue. Photomicrographs were taken on a microscope equipped with an Ibso microattachment and a Leica camera loaded with Kodacolor 35 mm. film.

APPENDIX V

TISSUE EMBEDDING PROCEDURES

MARAGLAS EPOXY RESIN (25)

The canal tissue was dehydrated in a graded ethanol series (40, 75, 95, and 100%) and embedded in maraglas epoxy resin according to the following.

Absolute ethanol, two changes of 15 minutes each

Propylene oxide, two changes of 15 minutes each

Propylene oxide and maraglas (1:1) for 1 hour at 25°C. and at least 6 hours at 4°C.

Propylene oxide plus maraglas (1:3) for 1 hour at 25°C. and at least 6 hours at 4°C.

Maraglas resin for at least 12 hours at 25°C. followed by at least 24 hours at 55-60°C.

The following embedding mixture produced a resin block with a hardness suitable for sectioning canal tissue.

Maraglas 655	70 parts
Diepoxide flexibilizer — DER-732	18 parts
Dibutyl phthalate plasticizer	10 parts
Benzyldimethylamine catalyst	2 parts

Canal complexes were mounted singly in tinfoil frameworks after the 1:3 propylene oxide-resin stage. Fragile tissue, such as that treated with potassium ferrocyanide and then washed, was prepared as described in the methacrylate section.

BUTYL METHACRYLATE

When the canal tissue was embedded in butyl methacrylate, suitable sections were obtained without extensive tissue distortion (Fig. 4). The canal tissue was

washed, dehydrated in a graded ethanol series, and embedded. Tissue agitation was minimized during the water and alcohol changes by gently transferring the liquids rather than the tissue, which was then mounted in the tinfoil framework and embedded. By minimizing tissue agitation, even material treated with potassium ferrocyanide and then washed, was easily embedded without disintegration. In order that the sections of canal tissue subjected to different chemical treatments could be compared, all material was embedded with methacrylate prepolymerized to the same degree.

MOUNTING IN THE TINFOIL FRAMEWORK

The tinfoil framework was designed to fit the BEEM polyethylene capsule (source — Polysciences, Inc., Rydal, Pennsylvania) used as an embedding mold (Fig. 35a). A canal complex was laid on the framework (Fig. 35b) and pinched between the two end flaps. No adhesive was necessary as tissue soaked in maraglas or methacrylate was sticky and stayed between the flaps.

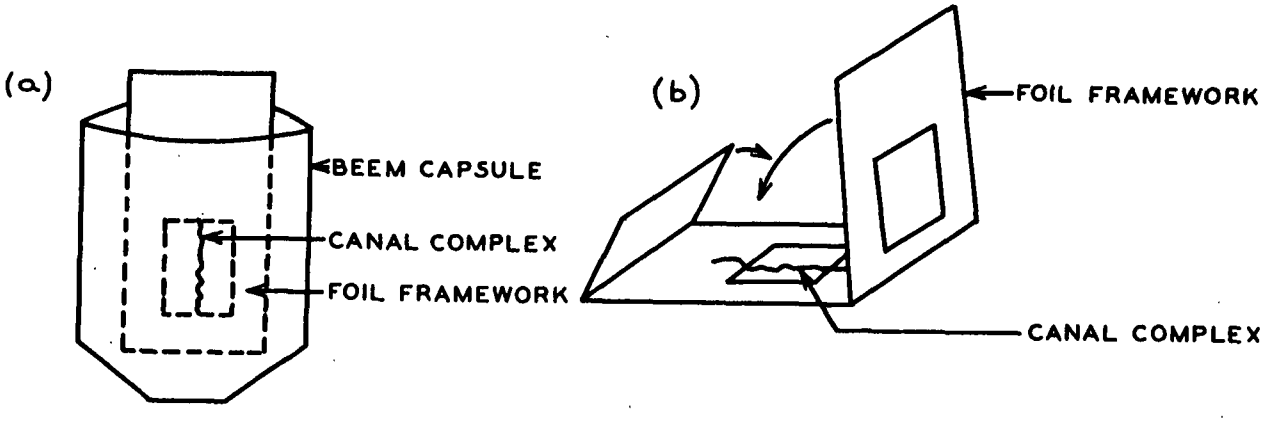

Figure 35. Mounting the Canal Complex in the Tinfoil Framework

APPENDIX VI

ELECTRON MICROSCOPY STAINS

METHODS AND EFFECTIVENESS

Two staining procedures were investigated, neither of which was completely satisfactory.

1. A procedure was designed which stained the canal complex before embedding by exchanging the calcium present in the tissue with the electron-dense uranyl (UO_2^{2+}) cation. The purpose was to put electron microscopy stains on a chemical as well as a contrast basis. Although a slight improvement in contrast was observed in comparison to the untreated canal tissue, it was not sufficient to consider further. The small amount of improvement was attributed to the fact that the canal complex was saturated with calcium (3.4%) which provided good contrast without further staining. This contrast was lost when the calcium and associated pectic substances were removed by acid or potassium ferrocyanide treatment. As most of the pectic substances were extracted, subsequent treatment in a uranyl acetate solution produced no improvement in contrast. Tissue contrast was attributed to cation exchange and not to precipitation of the uranyl cation.

2. Ultrathin sections of tissue treated in acid and potassium ferrocyanide and washed in water, were stained by the following procedure (27). Grids containing sections of tissue embedded in maraglas were floated, section side down, on a saturated solution of uranyl acetate for 2-4 hours. The grids were then dipped in water and allowed to dry before observation. A significant improvement in contrast was obtained, and the cellular outlines were at least identifiable. However, stained, untreated tissue showed only a slight improvement in contrast. Tissue contrast was attributed to precipitation of the uranyl cation and not to cation exchange.

The improvement in contrast achieved by staining ultrathin sections in uranyl acetate was not attributed to a chemical effect. Contrast within the cell wall was only slightly improved when the canal complex was soaked in uranyl acetate to replace cations already present. These observations suggest that the chemical significance of staining sections with a saturated solution of uranyl acetate is negligible. However, the vast improvement in contrast which this practice produced indicates that different potentials exist on the section surface which cause preferential precipitation. No evidence was obtained to suggest that barriers exist within the canal complex to prevent penetration and exchange of cations. Ultrathin sections, prepared from the canal complex after pretreatment with ferric chloride and treatment with potassium ferrocyanide, showed that both cell protoplasts and the cell wall were penetrated and stained.

APPENDIX VII

ANALYTICAL DATA FOR THE HOLOCELLULOSES AND THE CANAL TISSUE

TABLE VII

ANALYTICAL DATA FOR THE HOLOCELLULOSES AND THE CANAL TISSUE[a]

	Holocellulose[b]			Canal Tissue[b]		
	A	B	C	A	B	C
Rhamnan, %	ND	ND	ND	0.7	0.5	0.3
Araban, %	0.5	0.4	0.4	2.1	1.9	1.4
Xylan, %	4.2	3.9	4.3	4.6	4.7	5.2
Mannan, %	12.9	13.0	13.2	3.7	5.1	9.0
Galactan, %	1.6	1.0	1.2	3.9	4.3	2.5
Glucan, %	70.9	66.1	67.6	34.1	42.1	54.4
Uronic acid, %	4.5	5.1	NA	19.8	NA	NA
Galacturonic acid, %	0.4	0.4	0.3	17.1	13.1	6.2
Organic nitrogen, %	0.02	0.02	NA	0.08	0.14	NA
Klason lignin, %	ND	0.04	ND	NA	NA	NA
Methoxyl, %	NA	NA	NA	1.23	NA	NA
Acetyl, %	NA	NA	NA	ND	NA	NA
Ash, %	3.9	4.1	2.3	9.5	8.7	5.7
Silicon, %	0.006	0.04	0.008	0.02	0.12	0.29
Manganese, %	0.003	0.002	0.004	0.02	0.023	0.018
Iron, %	0.004	0.003	0.002	0.01	0.18	0.021
Lead, %	0.001	0.001	0.001	0.04	0.009	0.007
Calcium, %	0.64	0.60	0.3	3.4	2.6	1.86
Sodium, %	1.38	1.22	1.11	0.05	0.16	0.088
Aluminum, %	0.003	0.003	0.002	ND	0.03	0.012
Copper, %	ND	ND	0.001	0.02	0.016	0.002
Magnesium, %	ND	ND	0.002	0.008	0.046	0.012
Boron, %	ND	ND	ND	ND	ND	0.046
Chromium, %	ND	ND	ND	ND	ND	0.003
Nickel, %	ND	ND	ND	ND	ND	0.039

[a] Analyses made by the Analytical Department of The Institute of Paper Chemistry.

[b] A: Canal tissue from holocellulose A was used in the microscopic and cell separation studies.
B: Canal tissue from holocellulose B was used in the chemical composition studies.
C: Canal tissue from holocellulose C was used in the chemical composition studies. Sequestering stage omitted during canal complex purification.

ND — Not detected.
NA — Not analyzed.

TABLE VIII

ANALYTICAL DATA[a] FOR CANAL TISSUE B[b]

		Treatment						
		Ferric Chloride		Hydrochloric Acid		Potassium Ferrocyanide (pH 6.8)		
	Untreated	Residue	Residue Plus Extracts[c]	Residue	Residue Plus Extracts[c]	Residue	Washing Liquor Extract	Treating Liquor Extract
Rhamnan, %	0.5	0.6	0.2	0.3	1.2	0.6	0.4	0.2
Araban, %	1.9	1.5	ND	0.7	2.4	1.2	1.1	0.3
Xylan, %	4.7	4.2	0.04	4.2	0.8	5.4	1.1	0.1
Mannan, %	5.1	4.4	0.08	5.5	0.2	6.7	0.9	ND
Galactan, %	4.3	3.7	0.2	3.0	3.4	3.5	1.9	0.4
Glucan, %	42.1	42.2	1.5	47.1	2.1	58.8	2.2	0.6
Galacturonic acid, %	13.1	12.5	2.0	4.2	29.4	3.4	5.2	6.3
Organic nitrogen, %	0.14	NA	NA	NA	NA	0.13	NA	NA
Ash, %	8.7	7.5	NA	5.0	NA	9.3	55.1	67.1
Calcium, %	2.6	0.05	NA	0.22	NA	0.24	1.8	0.9
Iron, %	0.18	4.2	NA	0.06	NA	0.06	8.8	11.4
Sodium, %	0.16	ND	NA	0.18	NA	0.3	6.5	7.7
Potassium, %	ND	ND	NA	ND	NA	ND	29.8	25.8
Silicon, %	0.12	0.01	NA	1.3	NA	2.2	0.9	0.1
Other cations, %	0.08	0.02	NA	0.3	NA	0.3	1.9	0.1
Amount of tissue accounted for, %	74.9	73.4	NA	67.1	NA	82.8	NA	NA
Wt. of sample, g.	NA	0.093	0.0918	0.069	0.0227	0.190	0.0495	0.2385
Initial pH of liquor	NA	1.9	NA	1.9	NA	6.8	NA	NA
Final pH of liquor	NA	2.3	NA	2.4	NA	7.7	NA	NA

[a] Analyses made by the Analytical Department of The Institute of Paper Chemistry.
[b] Tissue B (Table II).
[c] Combined washing and treating liquor extracts.

ND — Not detected.
NA — Not analyzed.

The high ash and cation content in the potassium ferrocyanide extracts was attributed to decomposition of the ferrocyanide complex during the long dialysis period of 16 days. The ferrocyanide complex is only stable under alkaline and neutral conditions (72); therefore, decomposition was due to the slightly acid aqueous dialyzing medium (pH 5.5-6.5). Both extracts turned green during dialysis, apparently because ferricyanide-ferrocyanide complexes were formed (71, 72). These complexes did not pass through the dialysis tubing and were probably formed when ferrous ions from the unstable complex were converted to ferric ions by the acidic extracts. The small amount of iron in the tissue treated with potassium ferrocyanide showed that residual ferrocyanide was rapidly washed from the tissue before complex disintegration occurred.

The total glucan content (residue plus extracts equals 100 units) was the basis used to compare the different tissue residues (Table IX). This basis was not completely valid as some glucose was lost from the extracts during manipulation and dialysis. Consequently, the composition data of the residues are slightly high in relation to the untreated tissue. This discrepancy was unimportant since the residues were compared with the untreated tissue, and the errors involved were constant.

The poor rhamnan correlation between the untreated tissue and the residue plus extract was attributed to the small amounts involved. A similar explanation covers the discrepancy in the galacturonic acid content of the ferric chloride residue plus extract. When small quantities of a substance are analyzed (Table VIII), sampling and analytical errors are magnified.

The potassium ferrocyanide residue plus extract summations for galacturonic acid and calcium were very low compared with the untreated tissue. As galacturonic acid in the extracts was apparently reduced during oxidation of the decomposing

ferrocyanide complex, the low value was expected. However, the residue value was meaningful because these effects were absent. Although much of the calcium was dialyzed from the extracts, a small amount was trapped in the undialyzable ferricyanide-ferrocyanide particles.

TABLE IX

CANAL COMPLEX RESIDUE AND EXTRACT DATA[a] FOR CANAL TISSUE B[b]

	Treatment							
		Ferric Chloride		Hydrochloric Acid		Potassium Ferrocyanide		
	Untreated	Residue	Residue Plus Extracts	Residue	Residue Plus Extracts	Residue	Residue Plus Liquor Extract	Residue Plus Liquor and Washing Extracts
Rhamnan	1.2	1.4	2.0	0.6	1.8	1.0	1.4	1.6
Araban	4.5	3.4	3.4	1.5	3.0	2.0	2.6	3.1
Xylan	11.2	9.6	9.6	8.8	9.4	9.0	9.2	9.6
Mannan	12.1	10.1	10.3	11.5	11.8	11.1	11.1	11.5
Galactan	10.2	8.5	8.9	6.3	8.8	5.8	6.6	7.4
Glucan	100.0	96.6	100.0	98.5	100.0	97.8	99.0	100.0
Galacturonic acid	31.2	28.7	33.0	8.8	29.4	5.7	18.8	21.0
Organic nitrogen	0.3	NA	NA	NA	NA	0.2	NA	NA
Calcium	6.2	0.1	NA	0.5	NA	0.4	2.3[c]	3.1[c]
Iron	0.4	9.7	NA	0.1	NA	0.1	--[c]	--[c]

[a] Based on total glucose content (residue plus extracts equals 100 units).

[b] Canal tissue B (Table III).

[c] These values were meaningless as they were associated with ferri-ferrocyanide complexes (see Table VIII and pertinent discussion).

NA — Not analyzed.

APPENDIX IX

ANALYTICAL DATA FOR CANAL TISSUE C

TABLE X

ANALYTICAL DATA[a] FOR CANAL TISSUE C[b]

	Treatment				
		Hydrochloric Acid		Potassium Ferrocyanide (pH 6.8)	
	Untreated	Residue	Residue Plus Extracts[c]	Residue	Residue Plus Extracts[c]
Rhamnan, %	0.3	0.2	ND	0.3	ND
Araban, %	1.4	0.7	0.006	0.6	0.11
Xylan, %	5.2	5.1	0.05	4.9	0.05
Mannan, %	9.0	9.6	0.07	10.2	0.25
Galactan, %	2.5	3.2	0.09	2.0	0.18
Glucan, %	54.4	60.7	0.2	66.0	0.26
Galacturonic acid, %	6.2	2.9	NA	2.2	NA
Ash, %	5.7	1.06	NA	3.99	NA
Calcium, %	1.86	0.11	NA	0.12	NA
Iron, %	0.02	0.01	NA	0.04	NA
Sodium, %	0.09	0.16	NA	0.29	NA
Potassium, %	ND	ND	NA	ND	NA
Silicon, %	0.29	0.12	NA	0.90	NA
Other cations, %	0.14	0.11	NA	0.24	NA
Amount of tissue accounted for, %	81.4	82.9	NA	87.8	NA
Weight of sample, g.	NA	0.143	1.475	0.129	1.234

[a] Analyses made by the Analytical Department of The Institute of Paper Chemistry.

[b] Canal tissue C (Table II).

[c] Combined washing and treating extracts.

ND — Not detected.

NA — Not analyzed.

TABLE XI

CANAL COMPLEX RESIDUE AND EXTRACT DATA[a] FOR CANAL TISSUE C[b]

		Treatment			
		Hydrochloric Acid		Potassium Ferrocyanide (pH 6.8)	
	Untreated	Residue	Residue Plus Extracts	Residue	Residue Plus Extracts
Rhamnan	0.6	0.3	0.3	0.4	0.4
Araban	2.6	1.1	1.2	0.9	2.4
Xylan	9.6	8.1	9.0	7.2	7.8
Mannan	16.6	15.3	16.5	15.0	18.4
Galactan	4.6	5.1	6.7	2.9	5.4
Glucan	100.0	96.8	100.0	96.8	100.0
Galacturonic acid	11.4	4.6	NA	3.2	NA
Calcium	3.4	0.18	NA	0.18	NA
Iron	0.04	0.02	NA	0.06	NA

[a] Based on total glucose content (residue plus extracts equals 100 units).

[b] Canal tissue C (Table II).

NA — Not analyzed.

The high galactan content in the tissue treated with hydrochloric acid, relative to the untreated tissue and to the value listed in Table IX, could not be explained. The poor correlation between the residue plus extract and the untreated tissue was attributed to the small amounts of sugars detected in the extracts (Table X). A very small analytical error would cause these discrepancies.

APPENDIX X

ARTIFACTS CREATED DURING SECTIONING

The darkly stained tubelike structures (Ar) seen in the stained, ultrathin section in Fig. 8 are actually artifacts created during sectioning. This is proven in Fig. 24 where similarly labelled structures are visible in a shadowed, ultrathin section. The artifacts are actually folds in the ultrathin ($\approx$ 0.05 µm.) sections and are not structures which pass through adjacent cell walls. When sections of maraglas embedded canal complexes are stained with uranyl acetate, the heavy cation is concentrated in the folds making them visible [Fig. 8 (Ar)].